THE GENETIC ETIOLOGY OF EARLY-ONSET HEARING LOSS IN NEWFOUNDLAND AND LABRADOR

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Abstract

Hearing loss is the most common sensory disorder worldwide and > 50% of cases can be attributed to single gene mutations. I used a targeted candidate gene approach and Sanger sequencing to screen genomic DNA from 101 deaf probands with Newfoundland ancestry for pathogenic mutations in deafness genes. First I screened for mutations in *WFS1*, *TMPRSS3*, and *PCDH15* that were previously identified in this population, then for mutations in *Cx26* and *Cx30,* and mutations in the mitochondrial genes *MTRNR1* and *MTTS1*. Finally, genes were targeted based on patterns of hearing loss as seen on patient audiograms. Although several probands were "solved" by this approach, none had mutations in *WFS1*, *TMPRSS3* or *PCDH15*. One proband had digenic mutations in *Cx26* and *Cx30* and two probands inherited the A1555G mutation in *MTRNR1*. In order to decipher several variants of unknown pathogenicity and solve more families, further clinical recruitment and whole genome approaches are required.

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List of Abbreviations

Abbreviation	Meaning
AD	Autosomal Dominant
AR	Autosomal Recessive
ATP	Adenosine Triphosphate
bp	Base pair
bps	Base pairs
Cx26	Connexin 26
Cx30	Connexin 30
Cx31	Connexin 31
COCH	Cochlin gene
dB	Decibel
DFN	Deafness
DFNA	Nonsydromic deafness autosomal dominant
DFNB	Nonsyndromic deafness autosomal recessive
DIAPH1	Diaphanous-related Formin 1 gene
DNA	Deoxyribonucleic Acid
GJB2	Connexin 26
GJB3	Connexin 31
GJB6	Connexin 30
HDF	High-Dye Formamide
Hz	Hertz
KCNQ4	Potassium Voltage Gated Channel gene
mM	Milli-molar
mtDNA	Mitochondrial DNA
MTRNR1	Mitochondrially Encoded 12S RNA gene
MTTS1	Mitochondrial Gene, Encodes the transfer RNA for Serine
MYO6	Myosin VI gene
NL	Newfoundland and Labrador
NSHL	Nonsyndromic Hearing Loss
PCDH15	Protocadherin related 15 gene
PCR	Polymerase Chain Reaction
POU4F3	POU Class 4 Homeobox 3 gene
SHL	Syndromic Hearing Loss
TECTA	Tectorin Alpha gene
TMC1	Transmembrane Channel-Like 1 gene
TMPRSS3	Transmembrane Protease Serine 3 gene
µL	Micro Litre
µM	Micro-molar
WFS1	Wolframin Syndrome 1 gene

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1.Introduction

1.1 Purpose of Study

The objective of this project was to determine the underlying genetic cause of deafness in probands (affected individuals) from Newfoundland and Labrador. These individuals had been diagnosed with hearing loss. An attempt to identify genetic causes was carried out using modern molecular genetic techniques.

1.2 Overview of Hearing Loss

Hearing loss is one of the most common sensory disorders worldwide, affecting 1-3 in 1000 children at birth or in early childhood, and is caused by many known environmental and genetic factors (Retrieved from www.cdc.gov/ncbddd/hearingloss/ehdi-data2009.html on April 24, 2014). The prevalence of pre-lingual hearing loss increases if young children are included and all degrees of hearing loss are considered. Even hearing loss that follows environmental affliction (for example infection, acoustic trauma, or exposure to ototoxic drugs) is likely influenced by a genetic susceptibility (Ito *et al.*, 2010; Petersen *et al.*, 2006,;Nance, 2003; Keats & Berlin, 2002). The number of individuals affected by hearing loss increases as the population ages, with hearing impairment affecting 25% of individuals between the age of 50 and 65, and 50% of individuals over the age of 85 (Liu *et al.*, 2007). Like early onset hearing loss, late onset hearing loss can also be attributed to a variety of causes including environmental factors, medical disorders and their treatment, and genetic susceptibility (Liu *et al.*, 2007).

Hearing loss can be classified into three different types based on clinical presentation: conductive, sensorineural, and mixed – a combination of conductive and sensorineural. Conductive hearing loss results from abnormalities of the anatomic components of the ear that transfer sound waves to the cochlea, such as the external ear, the ear canal, ossicles (malleus, incus, stapes), tympanic membrane, oval window, round window, or middle ear space (Fig.1.1). Sensorineural hearing loss results from the dysfunction of the auditory pathway components that convert sound wave vibrations into an electrical impulse that is transferred to the auditory cortex in the brain. Therefore this type of hearing loss is caused by abnormalities within the cochlea, and/or auditory (cochlear) nerve, or in rare cases auditory brainstem, or auditory structures in the brain (Griffith, 2002) (Fig.1.1).

More than fifty percent of hearing loss cases can be attributed to an underlying genetic cause (Ito *et al.,* 2010; Norton 1991), and can be further categorized into two distinct groups: syndromic (SHL) or nonsyndromic (NSHL). When an individual presents with hearing loss along with other abnormalities, it is known as SHL. This occurs in approximately 30% of diagnosed individuals with over 400 hearing loss syndromes having been described, including Usher syndrome (hearing loss along with retinitis pigmentosa – loss of peripheral vision and night blindness), Pendred syndrome (hearing loss along with thyroid problems), and Jervell and Lange-Nielson syndrome (hearing loss along with cardiac dysrhythmia) (Van Camp *et al.*, 1997; Hilgert *et al.*, 2009). When hearing loss is not associated with any other abnormalities it is known as NSHL; 70% of hearing loss cases fall into this category (Hilgert *et al.*, 2009; Van Camp *et al.,* 1997).

NSHL can follow every known pattern of Mendelian inheritance including autosomal recessive (AR), autosomal dominant (AD), and X-linked. There are also mutations in the mitochondrial genome that result in non-Mendelian maternal inheritance, and NSHL. A set of nomenclature rules has been assigned to the different types of genetic deafness to classify them as AD, AR or X-linked (mitochondrial hearing loss does not have a specific nomenclature) and these types are named under the acronym DFN (from the word "deafness"). Following DFN are the letters 'A' or 'B', 'A' meaning AD inheritance (DFNA) or 'B' meaning AR inheritance (DFNB). When DFN is present without an A or B, it refers to X-linked deafness (Piatto *et al.*, 2009). AR inheritance means the individual has to inherit two copies of a mutated gene in order to be affected, this type of inheritance accounts for 77-88% of genetic NSHL. With AD inheritance, which accounts for 10-20% of inherited NSHL, the individual will be affected if only one copy of a mutated gene is inherited. The mutated gene can come from either parent, if one parent is affected each offspring has a 50% chance of inheriting the mutated gene, if both parents are affected the chance of inheritance increases to 75%. X-linked disorders are passed on from parent to offspring on the X chromosome and can be dominant or recessive, the most characteristic feature of these disorders is the absence of male-to-male transmission. X-linked disorders also severely affect males but have variable expression in females due to X chromosome inactivation, which occurs in early embryogenesis in female germ line cells to compensate for the X-linked gene dosage difference between males and females. X-linked inheritance accounts for 1-2% of NSHL. Finally, mitochondrial hearing loss is caused by a mutation in the genome of mitochondria, an

organelle found within all human cells, more frequently in cells that require an abundance of adenosine triphosphate (ATP), such as muscle cells, due to the fact that mitochondria generate most of the cell's supply of this form of chemical energy. Mitochondrial genetic disorders can only be passed down from a mother to her offspring because the mitochondria in an embryo come from the ovum. None of the mitochondria contained in the sperm are passed on to the offspring; therefore you will only see female-to-male and female-to-female transmission in mitochondrial genetics. Mitochondrial hearing loss is more variable and harder to detect than other types of NSHL, and accounts for 1-20% of NSHL depending on the population. (Nance, 2003, Petersen, 2012, Sirmaci, 2012) (Fig.1.2). When evaluating the frequency of NSHL and SHL within the different types of Mendelian inheritance, it can be shown that the most common type of hereditary hearing loss is nonsyndromic autosomal recessive.

In order to categorize hearing loss into specific modes of inheritance prior to genetic testing, two methods can be used, first a pedigree with a family history of a defined hearing loss diagnosis is required so the hearing loss can be traced throughout the family. Secondly, an audioprofile can be created using the person's behavioural audiogram and other pertinent information including age of onset and number of affected relatives. Use of these classification methods helps to narrow down the number of genes that are screened during the genetic testing phase, making the process more efficient.

For the first method, a pedigree is created using information from the affected individual and family members as well as medical and census records. The pedigree will show the relationships between individuals and their affection status. Even with a clear

family history, classification can be difficult, as some types of hearing loss have reduced penetrance. Penetrance is directly linked to variable expressivity, as both influence the effect that genetic mutations have on the population. Penetrance indicates the number of people who have a genetic mutation and display the disorder. Expressivity refers to the symptoms displayed by an individual that has a genetic disorder. More specifically variable expressivity is the phenomenon where different people have the same genetic mutation but display different symptoms or different severities of the same symptom. For example, hearing loss can have variable expressivity, meaning a number of individuals within a family have hearing loss but at different severities, some with a subclinical phenotype (i.e. a mild form of hearing loss that is undetectable by standard behavioural audiogram). In a study, ten individuals from a family are found to have a mutation in a deafness gene, though only six of these family members display hearing loss; this would be reduced penetrance, and it is common in AD disorders. It may make it difficult for a researcher to confirm that the mutation is the cause of the disorder. Given the fact that everyone has a different genetic background, a mutation can have a lowered penetrance, so low that it may not cause a detectable phenotype. Therefore an individual could have a mutation that causes hearing loss, but may not display it (i.e. variable expressivity). These types of cases make it difficult to consistently identify the mode of inheritance, especially in families with few affected individuals. Knowing the mode of inheritance is important because it helps to narrow down the list of candidate genes, as mutations in some genes are more likely to cause a specific type of hearing loss. AD disorders will often show up in every generation of a pedigree (called a vertical

inheritance pattern) and are transmitted from mothers or fathers to both sons and daughters. AR disorders show a horizontal pattern where they are generally seen in only one sibship, and usually the affected siblings will have unaffected parents. Like autosomal dominant inheritance, the number of affected females and males is nearly equal; meaning the chance of inheriting a mutation is not affected by sex. X-linked dominant disorders have a specific pattern where the disorder is passed to all daughters from an affected male and affected females will pass the disease to half of their sons and daughters. The crisscross pattern is often described as a "Knight's Move inheritance" after the move used in the game of chess. X-linked recessive disorders will appear in more males than females (due to the fact that males only have one X chromosome), and an affected male will not pass the disorder on to any of his offspring (although the females may be carriers) (Griffiths *et al.*, 1999). Mitochondrial disorders display a pattern of matrilineal (maternal) inheritance because the mitochondrial genome is inherited from the mother and not the father; therefore only children of affected females will potentially manifest the disorder, whereas offspring of an affected male will not be affected (Strachan *et al.*, 1999). Understanding the inheritance pattern can help when counseling families regarding prevention of the disorder.

The second method used to categorize hearing loss into specific modes of inheritance is audioprofiling, where several audiograms at different ages are plotted on the same graph. The different ages can be from a single individual or from different members of the same family (Smith *et al.*, 2013). A helpful online tool, called AudioGene (audiogene.eng.uiowa.edu)(Hildebrand *et al.*, 2008), was developed to aid in

audioprofiling, it "analyzes audiometric data and predicts the likely underlying genetic cause of hearing loss based on known phenotypic parameters" (Hildebrand *et al.*, 2008). The user inputs the proband's audiogram and age, and the same information for all affected relatives, then the tool will identify a list of candidate genes/loci based on this information. An audiogram is a graphic display of the softest sounds an individual can hear at each specific frequency. The frequency, the pitch of a sound, is measured in Hertz (Hz), and the intensity, the loudness of a sound, is measured in decibels (dB). The audiogram (Fig.1.3) is produced following a hearing test by an audiologist. The pattern of an audiogram can give an indication as to what type of hearing loss affects the individual (Retrieved from http://www.raisingdeaf kids.org/hearingloss/testing/ audiogram/ on August 23, 2013). On the audiogram, a reverse slope indicates low-frequency hearing loss, mid-frequency loss is shown as a dip in the middle of the graph, also known as a "cookie-bite". Loss of high and low frequencies is known as a "reverse cookie-bite loss" as the individual can hear better in the mid-frequency range creating a peak in the middle of the graph. High frequency hearing loss is also known as sloping loss due to the sloped configuration, and flat loss involves a similar hearing loss at all frequencies (Fig. 1.4) (Retrieved from http://www.hearinglosshelp.com/articles/ kindsofhearinglosses.htm on August 23, 2013). If a single pattern is present in all members of a family, it can predict the locus of the underlying genetic cause. For example the audioprofile of individuals with *WFS1* non-syndromic hearing loss has a highly characteristic shape (Fig.1.5) (Smith *et al.*, 2013).

By performing pedigree and audioprofile analyses the list of candidate genes can be greatly decreased. This helps the researcher narrow in on the underlying genetic cause of hearing loss before performing mutation screening.

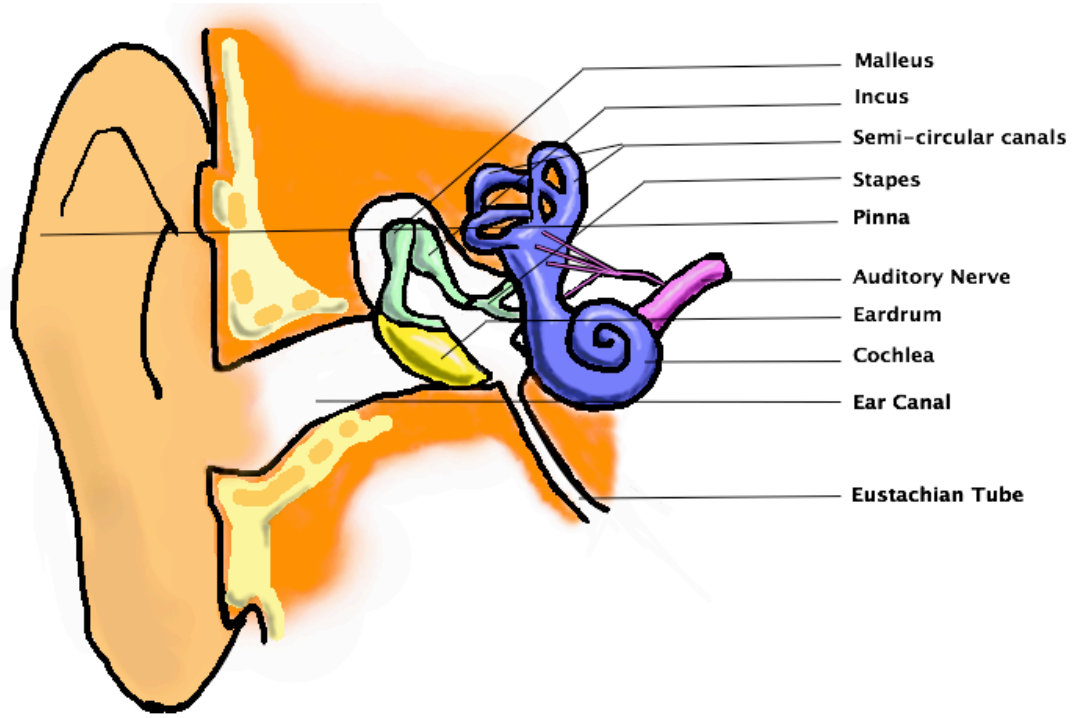


Figure 1.1. The anatomy of the human ear. (Created by author, adapted from http://commons.wikimedia.org/wiki/File:Ear-anatomy-text-small-en.svg)

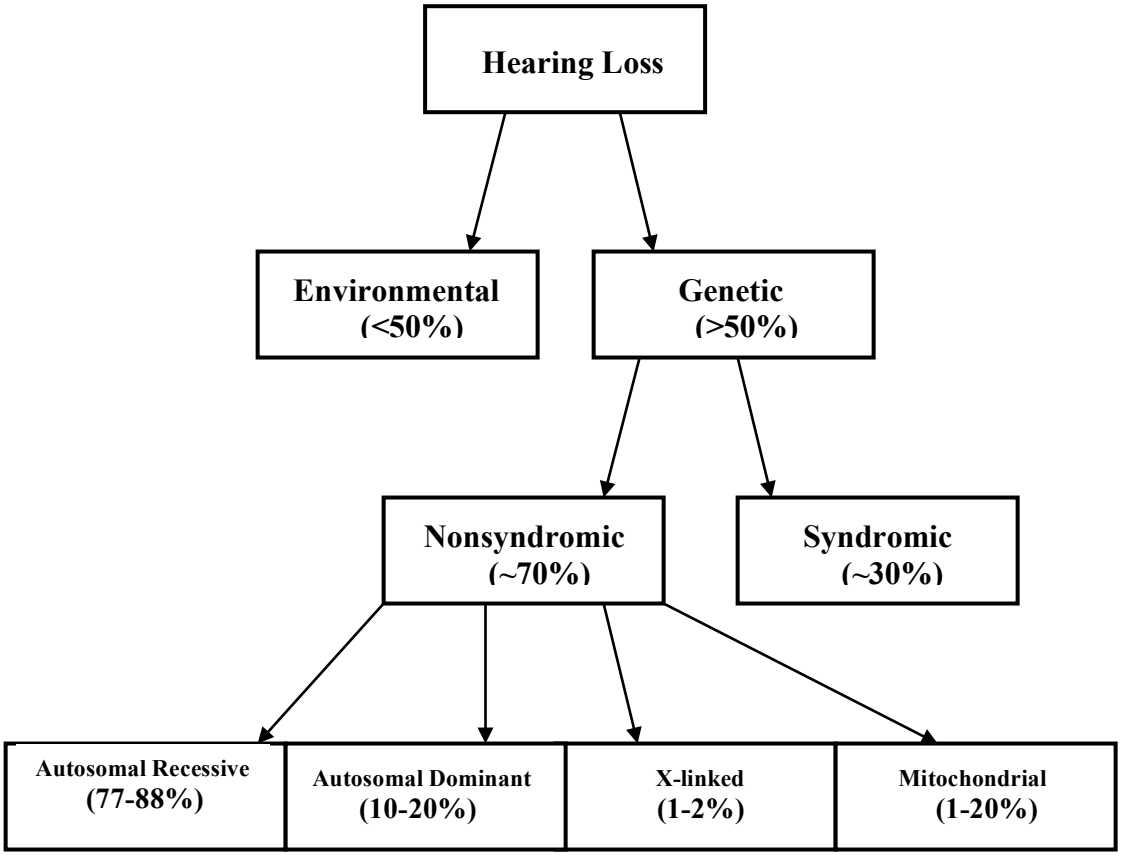


Figure 1.2. Flowchart outlining the causes of hearing loss

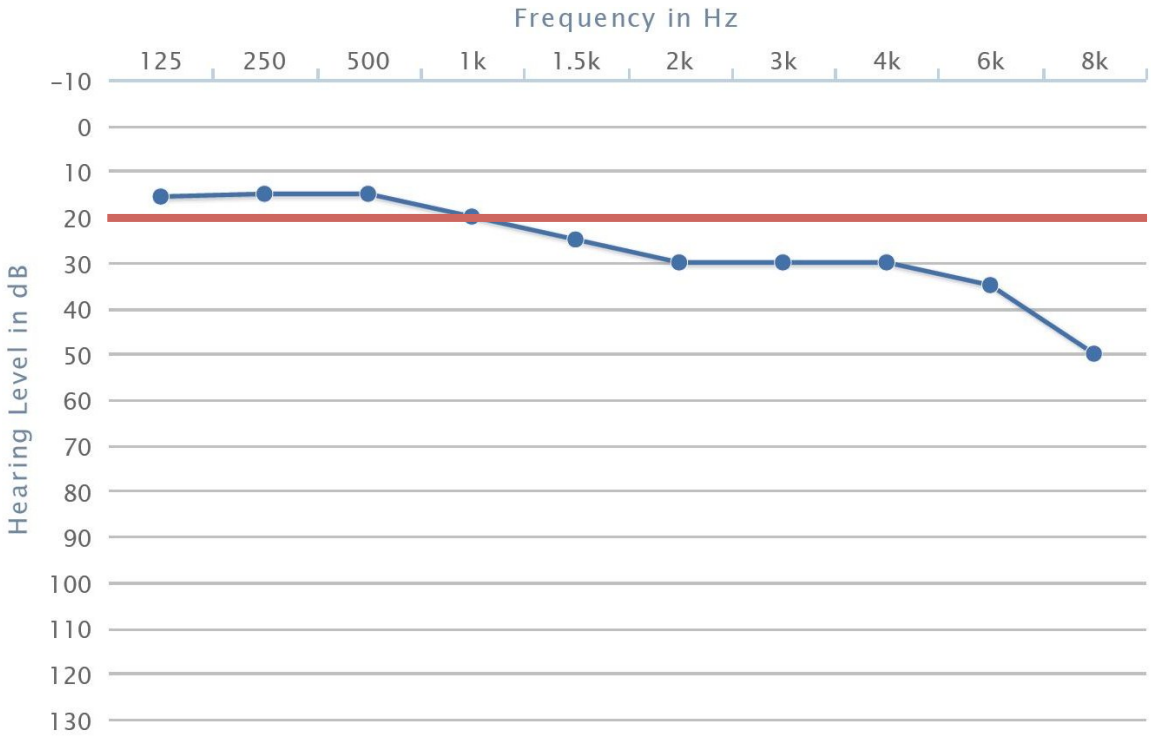


Figure 1.3. A graph of hearing threshold – to read this graph you look at the dots to see how loud a sound has to be at a specific frequency for the individual to hear it. For example at 8000 Hz the individual can only hear sounds louder than 50 dB. Therefore the person has trouble hearing high-pitched sounds, so has high frequency hearing loss. The red line indicates the threshold for hearing loss. When an individual cannot hear sounds above 20 dBHL, they are considered to have hearing loss at that frequency. This particular individual does not have hearing loss between 125 and 1000 Hz. (Image taken from http://audiogene.eng.uiowa.edu/audioprofiles)

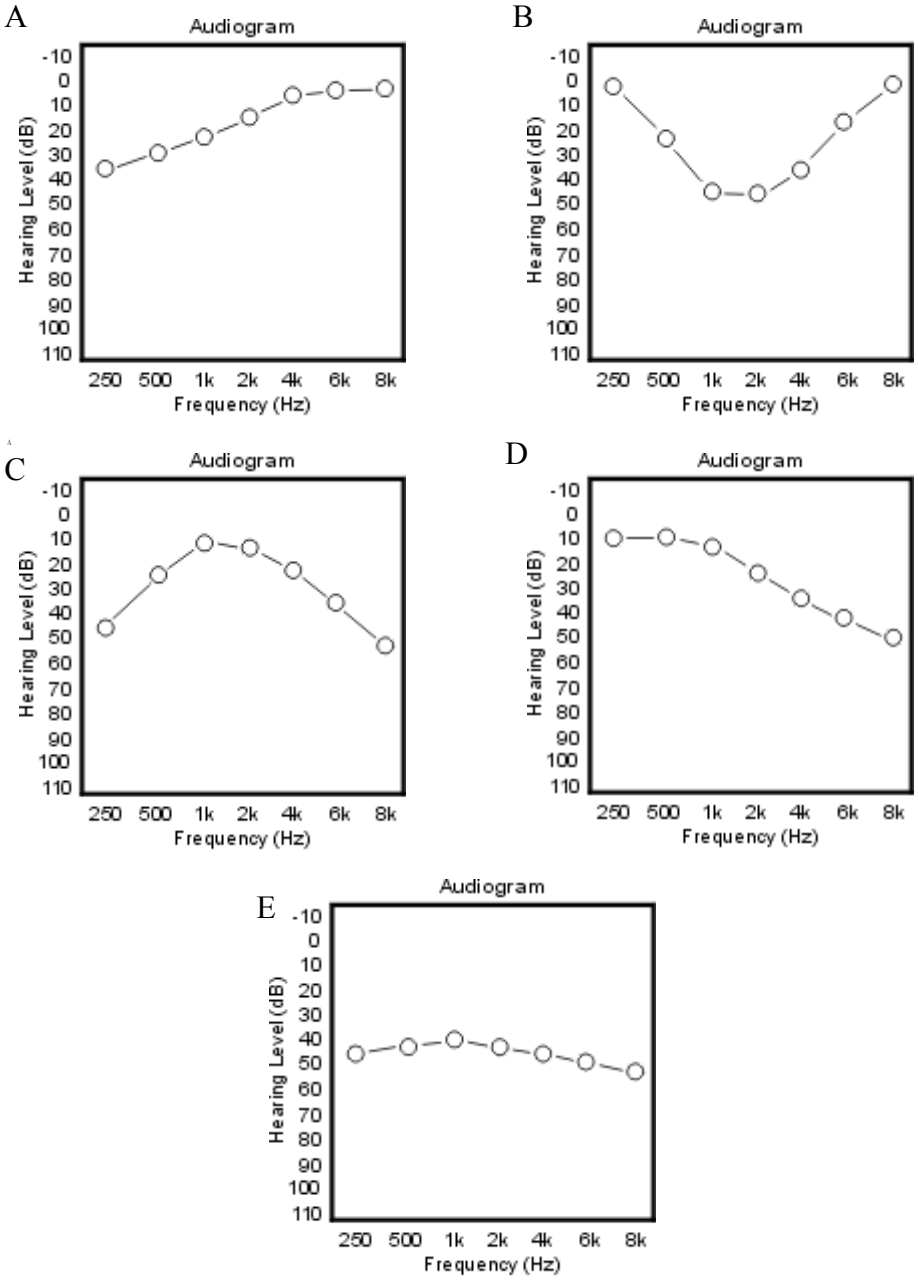


Figure 1.4. Sample audiograms. A – Reverse Slope, B – "Cookie-Bite", C- Reverse "Cookie-Bite", D – Sloping, E – Flat Loss
(Adapted from Center for Hearing Loss Help, http://www.hearinglosshelp.com/articles/kindsofhearinglosses.htm)

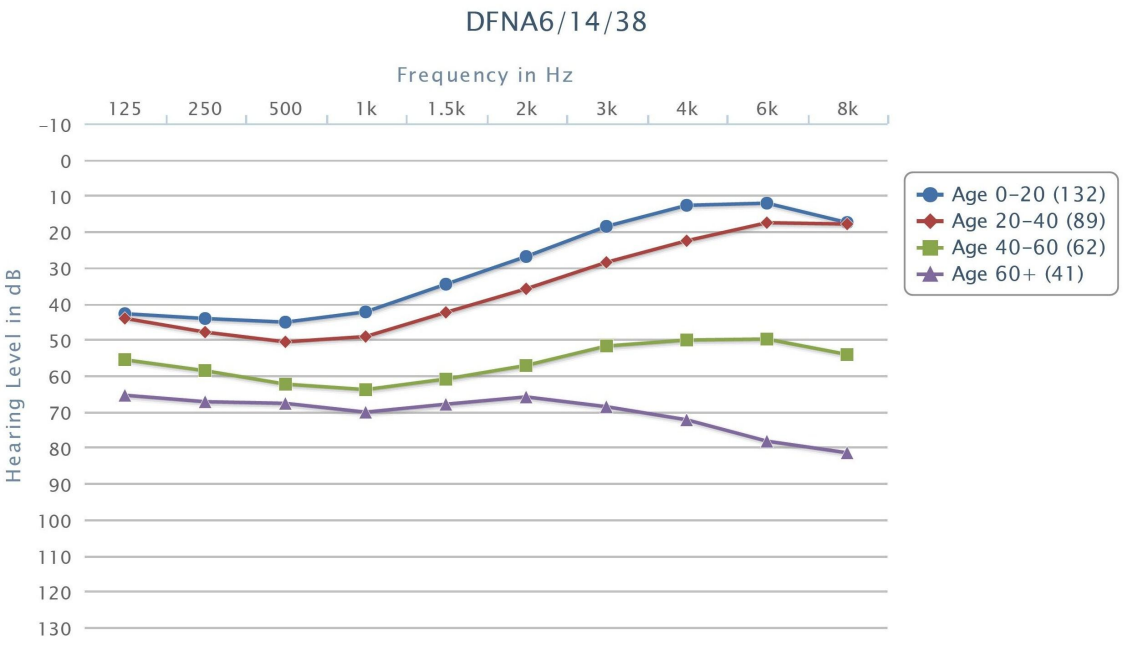


Figure 1.5. The characteristic audiogram of individuals affected by hearing loss often associated with mutations in the *WFS1* gene that causes nonsyndromic SNHL (DFNA6/14/38) (Hildebrand, M. S. *et al*., 2009)

1.2.1 Nonsyndromic Autosomal Recessive Hearing Loss

Hearing loss can be divided into SHL and NSHL subtypes. SHL is the diagnosis when hearing loss occurs with other clinical conditions or abnormalities. This occurs in approximately 30% of diagnosed individuals with over 400 hearing loss syndromes having been described, including Usher syndrome (hearing loss along with retinitis pigmentosa – loss of peripheral vision and night blindness), Pendred syndrome (hearing loss along with thyroid problems), and Jervell and Lange-Nielson syndrome (hearing loss along with cardiac dysrhythmia) (Van Camp *et al.*, 1997; Hilgert *et al.*, 2009). Nonsyndromic hearing loss is diagnosed when the loss of hearing is the only clinical phenotype. Nonsyndromic autosomal recessive hearing loss is generally prelingual (present before speech develops), almost exclusively a result of cochlear defects (thus sensorineural), and usually more severe than all other forms. This type of hearing loss is extremely heterogeneous; as of June 23, 2014, 80 loci have been mapped and 55 genes have been identified (Hereditary Hearing Loss Homepage http://hereditaryhearing loss.org). Like all autosomal recessive disorders an individual must inherit two mutated copies of the gene, one from each parent, to present with the disease phenotype. Mutations in genes that are located in the *DFNB1* locus, connexin 26 (*Cx26*) and connexin 30 (*Cx30*), are the cause of 30-50% of this type of hearing loss (Bhalla *et al.*, 2009). The remaining cases are due to mutations in numerous different genes (Hilgert *et al*., 2008).

Specifically within the *DFNB1* locus, mutations in *Cx26*, also known as *GJB2*, are the most common underlying cause of AR NSHL throughout the world (Kenneson *et*

al., 2002). Another connexin gene found within this locus and 29 kb away from *Cx26* is *Cx30* (or *GJB6)* and it is also responsible for many cases of this type of hearing loss. Both genes are found on chromosome 13 (13q.11-13.q12) (del Castillo *et al.*, 2002) and will be discussed in greater detail.

1.2.2 The Founder Populations of the island of Newfoundland

The population ancestry of Newfoundland (current population of 514,536 residents; Statistics Canada http://www.statcan.gc.ca/) is greatly influenced by natural expansion from early English and Irish settlers who came to fish the North Atlantic cod. The majority of people from Newfoundland are direct descendants of these first immigrants. The unique gene pool of this population is the consequence of a small number of founders and genetic drift, especially founder effect, a type of genetic drift that occurs when a small number of individuals break off from a larger population and form a new colony causing a reduction in genetic variation in the new population due to reproductive separation (Griffiths *et al.,* 2002). Many of the immigrants in the 1600s settled in coastal regions around Newfoundland to be in close proximity to the fishing grounds. Individual fishing villages, locally known as "outports", were isolated from each other by ocean and harsh terrain. Furthermore, marriages between Catholics and Protestants were strongly discouraged. The result was a high frequency of inbreeding within isolated communities which remained distant from each other, increasing the prevalence of specific genetic disorders such as hearing loss (Doucette *et al.*, 2009; Rahman *et al.*, 2003; Martin *et al.*, 2000). Other disorders, such as Familial Multiple Endocrine Neoplasia Type I (Farid *et al.*, 1980), hemophilia A (Xie *et al.*, 2002),

hereditary non-polyposis colorectal cancer syndrome (Froggatt *et al.*, 1999), and familial adenomatous polyposis (Spirio *et al.*, 1999) have been recognized to be due to founder effects in outport communities in Newfoundland. Extended, multiplex families with Newfoundland ancestry present an unprecedented opportunity to uncover the etiology of genetic diseases like hearing loss.

1.2.3 Recurrent Mutations: Founder Mutations vs. Mutation Hot Spots

Common mutations may be due to either founder mutations or to recurrent mutations at hot spots, therefore when a common mutation is discovered it is necessary to use haplotype analysis to figure out which type of mutation you have uncovered (Romdhane & Abdelhak, 2012). Founder mutations are mutations present in a high frequency in a particular population, and the reason for their existence is that the mutation was present in one ancestor or a small number of ancestors. Mutation hot spots can produce recurrent mutations because they are DNA sequences that are highly susceptible to be mutated (Retrieved from http://www.ncbi.nlm.nih.gov /books/ NBK5191/ on April 26, 2013). The high susceptibility of a sequence to be mutated can be caused by instability, unequal crossing over, or a predisposition to substitutions (Retrieved from http://www.ncbi.nlm.nih.gov/books/ NBK5191/ on April 26, 2013). To confirm that a common mutation is due to a founder effect, a method called haplotype analysis is carried out. A haplotype is the arrangement of marker alleles found on a single chromosome. Marker alleles can be single nucleotide polymorphisms (SNPs) or microsatellite markers (also known as short tandem repeats – repeating sequences of DNA) that are passed on together through a pedigree (Zhao *et al.*, 2003). Haplotype

analysis is a type of genetic test that looks closely at these linked DNA segments and shows how the specific genetic information is passed down through pedigrees (Retrieved from http://www.ncbi.nlm.nih.gov/books/NBK5191/ on April 26, 2013, Romdhane & Abdelhak, 2012). The mutation is considered a founder mutation only if it is located at a single haplotype. If the mutation is found on multiple haplotypes it is not a founder mutation, and may be due to a mutation hot spot (Romdhane & Abdelhak, 2012).

1.2.4 Previous Hearing Loss Studies in Newfoundland

The population of the island of Newfoundland has been the focus of extensive hearing loss research for more than fifteen years. In 2001, while studying a six-generation Newfoundland family, Young *et al.* identified a missense mutation (p. 2146 G→A) in the *WFS1* gene that was causing a dominant form of inherited progressive deafness. The mutation was found in all affected members of the family and the link between the mutations and hearing loss was supported by haplotype and mutation analysis. This study by Young *et al.* was the first to link a mutation in *WFS1* with nonsyndromic hereditary hearing loss (Young *et al.*, 2001). In 2004, Ahmed *et al.* discovered two recessive mutations in the *TMPRSS3* gene in a six-generation family from the South Coast of the island segregating an autosomal recessive form of early onset hearing loss. The role of *TMPRSS3* in hearing loss was first described in 1996 (Bonne-Tamir *et al.,* 1996; Veske *et al.,* 1996). Ahmed *et al.* first discovered that hearing loss in the Newfoundland family was linked to markers for *DFNB8/B10*. Sanger sequencing revealed two recessive mutations, within *TMPRSS3*, 207delC and IVS8 + 8insT, the latter being novel. Haplotype analysis revealed an apparent founder DFNB8/B10 associated haplotype. It

had previously been assumed that the founder effect of a mutation in the Newfoundland population would be high due to the isolation of the island. However, that was not the case in this study, which highlights the genetic heterogeneity of deafness, even in a founder population. As well, Ahmed *et al.*'s findings challenged the notion that the population of the island of Newfoundland was homogeneous.

A second (consanguineous) family, located on Newfoundland's South Coast, displayed profound hearing loss inherited as an apparent autosomal recessive trait. The underlying cause of this disorder was a novel mutation in *PCDH15* (a gene also associated with Usher's syndrome - a syndromic form of hearing loss associated with vision impairment, Ahmed, 2001), identified by Doucette *et al.* in 2009. In this study, a genome-wide scan was used to map the hearing loss trait to chromosome 10q21-22, and genotyping identified that individuals with a hearing impairment were homozygous for a 16Mb ancestral genotype that included 44 annotated genes. Sequencing analysis was performed and identified the novel V528D missense mutation in *PCDH15*. This is the second study that found a *PCDH15* mutation causing nonsyndromic hearing loss instead of Usher's syndrome *(*Doucette *et al.*, 2009). The first study in 2003, found two missense mutations (p.G262D and p.R134G) in *PCDH15* causing nonsyndromic recessive hearing loss (DFNB23) in two Pakistani families (Ahmed *et al.,* 2003).

1.2.5 Mouse Models and Hereditary Hearing Loss

It is difficult to follow the process involved in the formation of the human inner ear due to the lack of instruments available for observing the development, as well as the lack of cell lines that could be used to replicate the events involved (Chatterjee, 2011).

Animal models, particularly mouse models are ideal for studying inner ear development and also useful for researching human hereditary hearing loss (Friedman *et al.*, 2007; Vrijens *et al.,* 2008), because of the similarity between inner ear structures (Avraham, 2003). There are 77 deafness genes shared by humans and mice (Retrieved from http://hearingimpairment. jax.org/models.html on June 24, 2014). In addition to this, mice have a short gestational period, and large litter sizes make it feasible to produce large numbers of mutated mice (Avraham, 2003). Using mouse models, researchers can mutate a gene of interest, and then follow the development of the structures of the inner ear. Observing the resulting phenotype of the mutant mice provides insight into both the normal function of the gene as well as the negative impact of the mutation on hearing (Friedman *et al.*, 2007). For example, early studies by Avraham *et al.* (1995) discovered that the gene causing the deafness phenotype in the Snell's waltzer mouse is *Myo6*, providing a candidate gene to test in human subjects. *Myo6* encodes a myosin heavy chain protein that is expressed in the inner ear and is required for structural maintenance (Avraham *et al.*, 1995). Several years later, Melchionda *et al.* (2001) discovered the first human mutation in the human homologue *MYO6* to be associated with hereditary hearing loss.

1.2.6 Types of Mutations that cause Hearing Loss

Variants in DNA do not always result in a disease phenotype and instead can have a positive effect on an organism, giving them a survival advantage. When mutations do cause a disease phenotype they are called genetic disorders (Relethford, 2012). There are many different types of mutations that have different effects on amino acid codons, so

that genetic disorders caused by some mutations may be more severe than the disorder caused by other mutations in the same gene.

One type of variant, called a point mutation, is also known as a single base substitution because that is literally what occurs, a single base nucleotide is replaced with a different base nucleotide. There are three types of point mutations: neutral, missense, and nonsense. A neutral variant causes no change in the amino acid codon and therefore is not predicted to have a negative effect on the protein. A missense mutation occurs when the new (mutated) base nucleotide results in a new amino acid codon, for example, a change of A**U**A to A**C**A would change the codon from isoleucine to threonine. A nonsense mutation arises when the change of a base nucleotide results in a stop codon. If the codon for serine, U**C**G, was mutated to become U**A**G, this would be a nonsense mutation as UAG is a stop codon, meaning that this is the codon that signals the premature truncation of the protein with subsequent drastic effect on the phenotype.

Another type of mutation is a frameshift mutation; these can be deletions or insertions of one or more base pairs that can be detrimental when they occur within the coding sequence of genes. In a frameshift mutation the number of bases deleted or inserted is not divisible by three. For example, the deletion of five bases or insertion of two bases cause the reading frame to shift, altering the amino acid codons that follow the insertion or deletion and leads to premature truncation in most cases.

Other types of large multibase mutations include duplications – when a portion of a gene, or an entire gene, is reproduced, inversions – when a segment of a chromosome is flipped or reversed, and translocations – when segments of nonhomologous

chromosomes are interchanged. These can result in gene fusion where the piece of chromosome connects genes or parts of genes that would otherwise be separate. When these variants do not cause any noticeable effect on the organism's phenotype, they are said to be benign or nonpathogenic (Powar, 2007).

To determine the nature of the identified variant, first a literature search is conducted to locate the mention of the variant in previous studies, the second step is to check the variant in the publicly available SNP/1000 genomes database to determine the frequency of the variant in the disorder. If the identified variant was not detected in the SNP/1000 genome database, it is considered a novel variant. (Mitchell *et al.*, 2005). If the variant is considered novel, the researcher must confirm the pathogenicity of the mutation. To determine pathogenicity of a mutation, it is helpful to have access to the DNA of other family members, affected and unaffected. By sequencing the DNA of the family members, a researcher can get clues as to whether or not the identified variant is pathogenic. If the variant is found in all affected family members but not in the unaffected members, this is one piece of evidence that the mutation may be causative. If the mutation is found in all or many family members regardless of their affection status, then it is likely that the mutation is benign. The next step would be to check for the mutation in the general population, usually by screening members of the same ethnicity. If the variant is not found in a general population screen, and is found in only affected members of the family, it is very likely the variant is a causative mutation. If a high proportion of the population does have the mutation, it is unlikely to be pathogenic.

Another piece of the puzzle is to use prediction programs to determine the impact of a variant on the protein. There are several prediction programs available to check the probability of a mutation being pathogenic. Such programs include SIFT (retrieved from http://sift.jcvi.org/ on March 20, 2012) which predicts whether an amino acid substitution affects protein function, WebLogo (retrieved from http://weblogo.berkeley.edu/ on March 20, 2012) which generates sequence logos showing sequence conservation at specific positions, PolyPhen-2 (Retrieved from http://genetics.bwh.harvard.edu/pph2/ on March 20, 2012) which predicts the impact of an amino acid substitution on the structure and function of the protein, and PMut (Retrieved from http://genetics.bwh.harvard.edu/pph2/ on March 20, 2012) which predicts the pathogenicity of a mutation based on the protein sequence. Using these programs can help determine the pathogenicity of a variant.

1.2.7 Cochlear Function

Mutations in the *Cx26* gene, within the *DFNB1* locus on chromosome 13, cause a large proportion of NSHL; the percentage varies depending on the population (Connexin-Deafness Homepage). In order to discuss the role of connexins in hearing loss, it is necessary to understand the function of the cochlea. Function of the cochlear tissues and auditory nerve depends on the ionic environment surrounding them within the inner ear, more specifically the concentration of potassium, an ion with an important involvement in sensory transduction (and all nerve signals throughout the human body). Three fluid filled spaces in the cochlea, the scala media (containing endolymph), the scala tympani and vestibule (both containing perilymph), are separated by tight junction barriers

(Fig.1.7). The scala media is filled with potassium rich endolymph whereas the scala tympani and vestibuli are filled with the sodium rich perilymph, with a composition very similar to intercellular and extracellular fluid ion concentrations, respectively. Sensory transduction is controlled by the endocochlear potential (EP), which is approximately 80 millivolts (mV) and is generated and maintained by the stria vascularis. The distinct ion concentrations in each of the fluid filled spaces are maintained by the tight junction barriers between them (Wangermann, 2006).

The sensory receptors within the inner ear are called hair cells (Fig.1.4). The hair cell stereocilia are immersed in endolymph, while the cell body is surrounded by perilymph. When a sound travels through the outer ear, it vibrates through the ossicles and moves the endolymph and basilar membrane upon which the hair cells sit. The receptor potentials are generated by the movement of potassium from the endolymph into the hair cells via the stereocilia. This begins a cycle where the potassium moves out of the hair cell body into the perilymph and eventually returns to the endolymph via the stria vascularis (Kikuchi *et al*., 2000) (Fig. 1.7).

1.2.8 Connexin Hearing Loss

Connexins are gap junction proteins that regulate ion concentrations and are found in many different vertebrate tissues. Daniel Goodenough proposed the name connexin in 1974. Before Goodenough isolated the subunit and called it a connexin, J. David Robertson hinted at a hexagonal lattice of subunits in 1963 when he isolated the subunits from Mautner cells (cells that make up the neural circuit in teleost fish that

mediate an escape response) of the common goldfish. He found what he called "honey-comb-like hexagonal arrays of closely packed subunits" which Goodenough (1975) later called connexons (the assembly of six connexin proteins) when he investigated the structure of the gap junction of mice hepatocytes using electron microscopy, biochemistry, and x-ray diffraction techniques. The idea that gap junctions are made up of subunits, connexons, which further consisted of smaller units called connexins wasn't actually confirmed until 1977 when Makowski and colleagues used x-ray diffraction to elucidate the composition of gap junctions (Makowski *et al.*, 1977). They established that the connexon was in fact a hexamer composed of molecular protein units (connexins) that had a molecular weight between 23-28 kilo Daltons (kDa) and that the connexons in plasma membranes of one cell connect to connexons in membranes of an adjacent cell to form the gap junction (Fig.1.6).

The human genome contains 21 different connexin genes (Retrieved from http://omim.org/ on August 14, 2011). The first connexins to be named were connexin 32 (*GJB1* OMIM# 304040) and connexin 43 (*GJA1* OMIM # 121014) (Beyer *et al*., 1987). The genes were first named for the molecular mass of their protein, for example, connexin 32 is approximately 32 kDa , connexin 43 is approximately 43 kDa, etc. This method for nomenclature was used for a long time and is still in use today. However, once connexin genes were separated into alpha and beta groups based on sequence similarities and the overall predicted topological organization (Gimlich *et al*., 1990), a new type of classification was established. Those belonging to the alpha group were called "A" and those belonging to the beta group "B". In addition to this distinction they

were called GJ, for gap junction, and given a number identifying the order in which they were discovered, e.g. *GJA1* is connexin 43.

Each of the 21 known connexin proteins has been mapped to different areas in the human genome (Retrieved from http://omim.org/ on August 14, 2011). These genes are developmentally regulated and can be co-expressed in the same cell (Kumar *et al*., 1992). The function of many of the connexin genes and their products has been revealed through the study of the effect of mutations within the genes. Mutations in many of these genes have been associated with disease. For example mutations in the connexin 32 gene, *GJB1,* underlie Charcot-Marie-Tooth-Disease, a neurological disorder involving weakness in the foot and lower leg as well as difficulty with auditory and fine motor skills (Bergoffen *et al*., 1993). Mutations in more than one connexin gene (Cx 26 and Cx 30) also cause nonsyndromic hearing loss (Pfenniger *et al*, 2010).

Within the cochlea both *Cx26* and *Cx30* are components of gap junction systems, which are essential to the cycling of potassium. They form the channels that facilitate the constant movement of potassium ions through the hair cells and back into the endolymph, an ionic current flow that is undulated by vibration during the transduction of sound into an electrical signal (Carlsson *et al.*, 2012; Irshad *et al.*, 2012; Ru *et al.*, 2012).

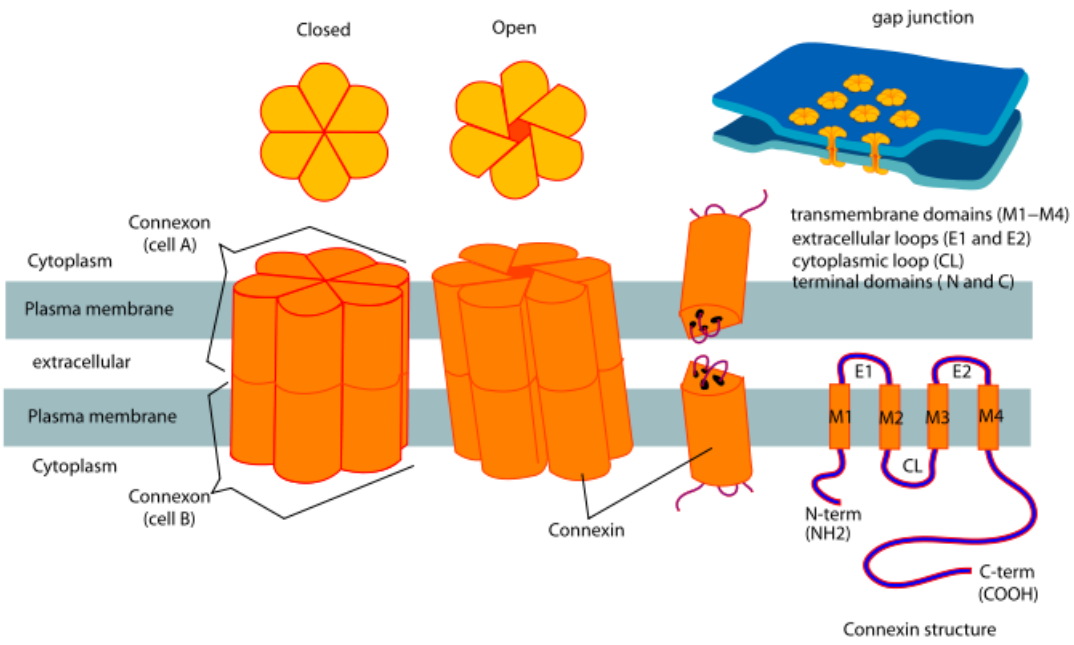


Figure 1.6. Six connexins come together to form a connexon. Connexons are located in the cell membranes of cells. When cells come together connexons meet to form gap junctions. (Image taken from http://commons.wikimedia.org/wiki/File :Connexon_and_connexin_structure.svg)

1.2.8 Connexin 26 and Hearing Loss

Kelsell *et al*. identified *Cx26* as the first autosomal NSHL susceptibility gene in 1997. The authors realized that the *DFNB1* locus and *Cx26* mapped to the same location on chromosome 13. When they sequenced *Cx26* in individuals with a hearing impairment from *DFNB1*- linked Pakistani families, they discovered homozygous substitutions in two of the hearing impaired offspring and found their unaffected parents to be heterozygous carriers for these mutations. Kelsell *et al.* also identified mutations in the *Cx26* gene in other affected members of different Pakistani families, giving them further

proof that deafness in these families was caused by mutations in *Cx26*. The authors took this to mean that the *Cx26* protein is an essential component of the cochlea. We now know that *Cx26* is expressed in many areas within the cochlea, specifically in the regions that separate the scala media from the scala tympani (Retrieve from http://hereditaryhearingloss.org on November 23, 2011) (Fig.1.7). The loss of the function of *Cx26* within the inner ear is expected to disrupt the flow of potassium thus interrupting the generation and passage of the electric signal from the inner ear to the auditory nerve and onto the brain (Petersen 2012).

As of June 27, 2014, 111 NSHL mutations have been described within the *Cx26* gene, including splice, nonsense, missense, and frameshift mutations (Retrieved from http://davinci.crg.es/deafness/ on June 27, 2014). The most common *Cx26* mutation in the Caucasian population (Denoyelle *et al.*, 1997), the deletion of a guanine at position 35 (c.35delG), was first identified by Zelante *et al*. in 1997 after they sequenced the *Cx26* gene in thirty-five affected patients from the Mediterranean. This mutation causes a frameshift that leads to premature chain termination and is found in a region of six guanine nucleotides close to the 5′ end of the *Cx26* coding region (Zelante *et al*., 1997). Most of the mutations in *Cx26* lead to malformed channels, which causes altered assembly of hemichannels, disrupted targeting to the plasma membrane, or decreased protein stability (Thönnissen *et al*., 2002).

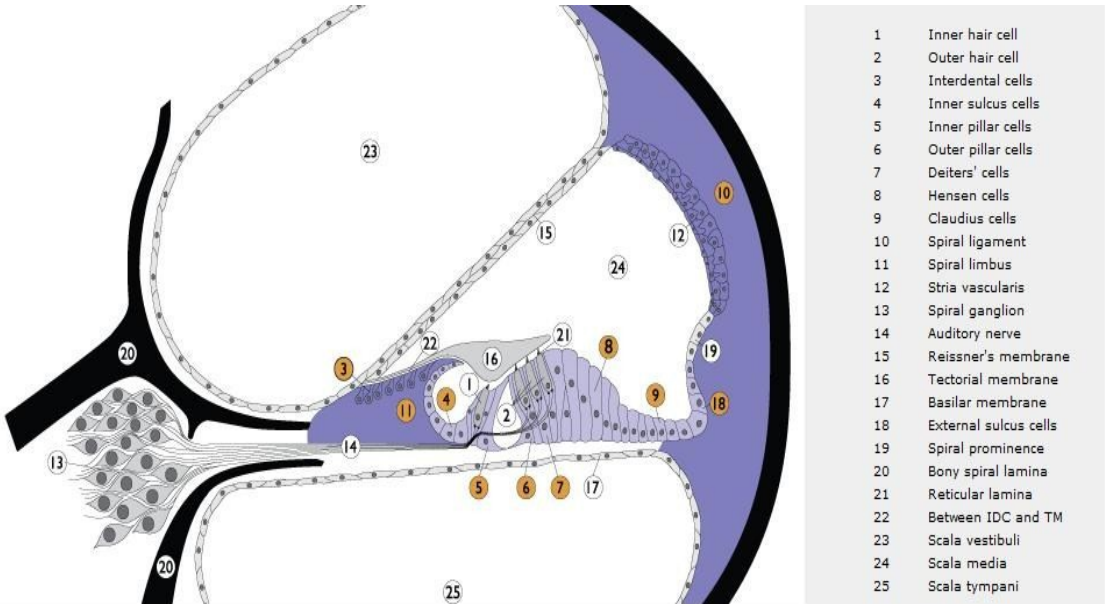


Figure 1.7. A drawing of the inner cochlea highlighting the areas in which connexin 26 is expressed. The numbers shaded orange are the areas of expression. These are the same areas of expression as connexin 30. (Image taken from Van Camp G, Smith RJH. Hereditary Hearing Loss Homepage. http://hereditaryhearingloss.org)

1.2.9 Connexin 30 and Hearing Loss

Cx30 (*GJB6*) is another connexin gene heavily involved in NSHL and is associated to hearing loss caused by mutations in *Cx26* (retrieved from davinci.crg.es/deafness/ on November 24, 2011). *Cx30* was first described in the *DFNA3* locus and found to cause autosomal dominant hearing loss (Grifa, 1999). It was discovered when 38 families, with hearing loss linked to chromosome 13, tested negative for *Cx26* mutations (Grifa, 1999). *Cx30* maps to chromosome 13q12 and is located near (~800kb) connexin 26 (Grifa, 1999). Close to 50 percent of individuals who are

heterozygotes for *Cx26* mutations also have a large deletion in the *Cx30* gene causing hearing loss (del Castillo *et al.*, 2003; Mahdieh *et al.*, 2010)

Many hearing impaired patients with nonsyndromic hearing loss (NSHL) have only one mutated allele in *Cx26* (del Castillo, 2002). For a long time these individuals were considered unsolved because another *Cx26* mutation was suspected, but could not be found. Even more confounding was that some patients with hearing loss showing linkage to *DFNB1* were void of mutations in *Cx26* (del Castillo, 2002). In 2002, del Castillo *et al*. discovered a 342kb deletion, which they later named Δ(*GJB6*-D13S1830), in the *Cx30* gene - a gene known to be co-expressed with *Cx26* in the inner ear (Fig.8). This discovery was made in 33 unrelated probands affected by hearing loss with heterozygous mutations in *Cx26*. Haplotype analysis was performed using markers on 13q12 to look for mutations outside of *Cx26*. Del Castillo *et al.* found that it was impossible to amplify several markers in the vicinity of *Cx30* in two of the subjects, which led them to believe that the subjects harboured a germline deletion. Upon analysis of DNA fragments of *Cx30* they discovered that the deletion truncated the open reading frame of the gene. The authors were able to determine the break points using sequence tagged sites, and they created a specific Polymerase Chain Reaction (PCR) assay to detect the Δ(*GJB6*-D13S1830) deletion (del Castillo, 2002). PCR is a technique that uses DNA polymerase to synthesize a new strand of DNA that is complementary to the template strand added to the reaction mixture. At the beginning of the reaction the mixture is heated to separate the strands of DNA. DNA polymerase then synthesizes the complementary strand between specific primers. The mixture is then cooled and the DNA

anneals. The cycles of heating and cooling are repeated, thus causing exponential amplification of the targeted section of DNA. When the process is completed this section of DNA will have accumulated in billions of copies. The *Cx30* deletion truncates the protein and is often found in conjunction with a heterozygous mutation in *Cx26* in hearing loss patients. The discovery of Δ(*GJB6*-D13S1830) solved a portion of the hearing impaired cases that were unexplained up to this point.

Once del Castillo *et al*. discovered this deletion they questioned whether the pattern of inheritance, where deafness was caused by two heterozygous mutations in two different genes, was monogenic or digenic (del Castillo *et al.*, 2002). If it were a monogenic pattern (due to a single gene), there would need to be a regulatory element upstream of both genes that controlled the expression of *Cx26*. They reasoned that the deletion would suppress gene expression, resulting in hearing loss (del, 2002). A second explanation would be that the deletion disrupts the activity of another gene that is important for the function of *Cx26*. Evidence suggests that *Cx30* is this other gene that is required for *Cx26* to function normally (del Castillo, 2002). Del Castillo *et al.* (2002) suggested that the *DFNB1* locus contains two genes, *Cx26* and *Cx30*, and altering any two of the four alleles will cause hearing loss. Pallares-Ruiz *et al.* (2002) also suspected that this phenomenon represented a pattern of digenic inheritance and tried to determine if this was the fact, however their results were inconclusive. It was not until 2009 that a group provided conclusive results after testing this hypothesis. Rodriguez-Paris *et al.* (2009) used allele specific analyses based on reverse transcriptase PCR (RT-PCR) using buccal cells that express both *Cx26* and *Cx30* equally. In the three hearing impaired

probands studied, all of whom carried Δ(*GJB6*-D13S1830), none expressed *Cx26* when *Cx30* had been altered, proving that the *Cx26* protein is not produced when Δ(*GJB6*-D13S1830) is present in the *Cx30* allele. This finding would be expected with the loss of a *cis*-regulatory element found within the deleted region thus disrupting the transcription of *Cx26* (Rodriguez-Paris, 2009).

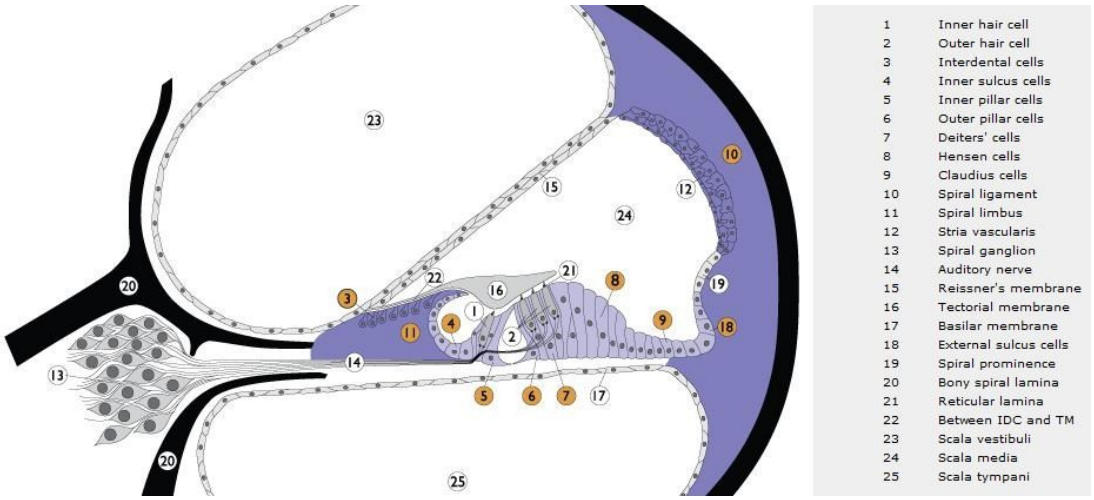


Figure 1.8. A drawing of the inner cochlea highlighting the areas in which connexin 30 is expressed. The numbers shaded orange are the areas of expression. Again, notice that the areas of expression are the same for connexin 30 and connexin 26. (Image taken from Van Camp G, Smith RJH. Hereditary Hearing Loss Homepage. http://hereditaryhearingloss.org)

1.2.10 Connexin 31 and Hearing Loss

Cx31, located on chromosome 1p34, encodes the human gap junction protein β-3, also known as *GJB3*, and is expressed in the skin, inner ear (specifically the spiral ligament, spiral limbus, and the auditory nerve), and the auditory nerve (Fig.1.9). Like

connexin 26 and 30, connexin 31 forms channels called gap junctions in the inner ear that allow the movement of potassium ions between the organ of Corti and the endolymph of the scala media (Hauwe *et al.,* 1999; Oh *et al.,* 2012).

Linkage of *Cx31* to hearing loss was first shown in 1998 when Xia *et al.* explored the possibility that other connexin genes (besides *Cx26* and *Cx30*) might be involved in the hearing process. They used two multiplex families of Chinese ancestry to map the third connexin gene to chromosome 1p33-p35, and used Sanger sequencing to reveal two mutations, a missense (c.547 G>A: p.Q183K) and a nonsense (c.538 C>T: p.R180X) mutation associated with autosomal dominant, bilateral, high-frequency loss. Using RT-PCR they showed that *Cx31* was expressed in the rat inner ear.

Mutations in *Cx31* have also been shown to cause recessive hearing loss in a compound heterozygous manner (Liu *et al.*, 2000). Liu and his team of researchers looked at 25 Chinese families with recessive hearing loss and found that the members of two of the families, affected by early onset, sensorineural, bilateral hearing loss, had an in-frame 3 base pair deletion (423-425ATT) in one allele causing the loss of an isoleucine residue at codon 141 and a c.423A>G transversion (p.I141V) in the other allele. Therefore, similar to *Cx26*, mutations in *Cx31* can cause either autosomal dominant or autosomal recessive hearing loss (Liu *et al.*, 2000).

In 2000, López-Bigas *et al.* found five SNPs (c. 1227C>T, c.1610G>A, c.1700 C>T, c.1731G>A, and c.1931C>T) in *Cx31* in patients affected by hearing loss. Two of the SNPs, c.1227C>T and c. 1731G>A, cause amino acid changes p.R32W and V200I, respectively. The nucleotide change c.1931C>T was found in 24 of the 153 hearing

impaired subjects screened, however it was also found in 13 percent of the control population. In much the same way p.R32W was found in seven of the 153 subjects with hearing loss, but also in 18 percent of the control population. The other three changes were not found in the ethnically matched control population; however the amino acid change V200I is in an area that is not well conserved, and in the rat and mouse the amino acid at position 200 is an isoleucine rather than a valine. The other two SNPs (c.1227C>T and c.1610G>A) had no obvious effect on the protein, thus López-Bigas *et al.* concluded that none of the SNPs found were causative, but would be useful in segregation and linkage disequilibrium analysis which can determine the patterns of inheritance.

Rouan *et al.* (2003) used HeLa cells to determine the function of the *Cx31* protein with a R32W variant. In this case they were investigating the connection between this variant and erythrokeratodermia variabilis (EKV), a skin disease that is also caused by mutations in *Cx31*. They transfected HeLa cells with mutated and wild type *Cx31* expression constructs and looked at synthesis, intracellular distribution, and protein assembly. The cells with mutated *Cx31* showed no deviations in expression level, connexon assembly, or intracellular distribution. They concluded that R32W is an inconsequential sequence polymorphism of *Cx31* (Rouan *et al.*, 2003).

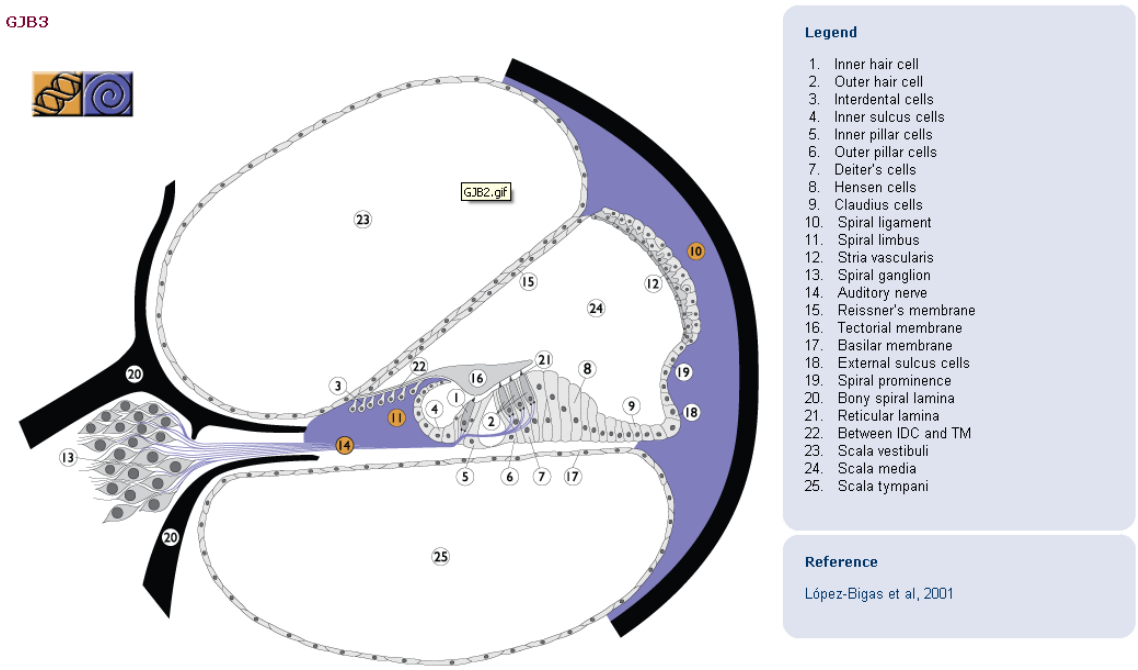


Figure 1.9. A drawing of the inner cochlea highlighting the areas in which connexin 31 is expressed. The numbers shaded orange are the areas of expression. (Image taken from Van Camp G, Smith RJH. Hereditary Hearing Loss Homepage. http://hereditaryhearingloss.org)

1.2.11 Digenic Inheritance and the Connexin Genes

It is now an accepted fact that mutations in two separate loci (genes) can cause recognisable patterns of hereditary deafness, known as digenic inheritance. For example, in one study it was shown that non-syndromic hearing loss can be caused by digenic inheritance of mutations in *Cx26* and *Cx31* (Liu *et al.,* 2008). This group of researchers screened the *Cx31* gene in 108 patients from China who were already shown to have heterozygous *Cx26* mutations and who did not have mutations in *Cx30*. They found two mutations (N166S and A194T) in *Cx31* in patients who had either 235delC or 299delAT mutation in *Cx26*; none of the *Cx31* mutations were found in the Chinese population controls. Because *Cx26* and *Cx31* have overlapping expression patterns it is likely that

these proteins have direct physical interaction (Liu *et al.,* 2008). They also found that *Cx26* and *Cx31* form heteromeric connexons in mouse cochlea leading them to conclude that the mutations they found in *Cx26* and *Cx31* were in fact the cause of hearing loss in the patients involved (Liu *et al.,* 2008).

Recently Oh *et al.* (2012) looked for a relationship between variants in *Cx31* and *Cx30* and nonsyndromic hearing loss in the Korean population. Through gene sequencing they found a total of nine variants, four of which were novel. They chose five of these variants (three novel: *Cx31*: V27M and V43M, *Cx30*: A40V, two known: *Cx31*: V84I, *Cx30*: A40V) to perform functional studies using a pathogenicity prediction program, also the area of the mutation was analyzed for conservation (other variants were excluded due to the frequency of their occurrence in unaffected individuals). Three of the variants, V27M and V84I in *Cx31,* and A40V in *Cx30*, were predicted to be deleterious because they were not found in unaffected individuals and the residues were highly conserved among different species (Oh *et al.*, 2012). As well, using biochemical-coupling tests, two of the variants in *Cx31* (V27M and V84I) were shown to affect protein function when they were present in a heterozygous form with wild type *Cx31*. The biochemical-coupling tests were performed by recording the time it took to diffuse Lucifer Yellow dye from cell to cell using cells that contained connexins with heterozygous mutations and WT mutations. The times were recorded as either immediate transfer: less than 30 seconds, delayed transfer: 30 seconds to 3 minutes, and no transfer: more than 3 minutes. If the time was delayed or there was no transfer then it was inferred that the protein was not functioning correctly (Oh *et al.*, 2012).

The studies discussed suggest that *Cx31* plays a major role in the hearing process and mutations in this gene can cause hearing loss in both an autosomal dominant and autosomal recessive manner. It has also been suggested that this gene be included in hearing loss screening studies along with *Cx26* and *Cx30* because of the connection shown between these connexin genes and hearing loss.

1.2.12 Mitochondrial Hearing Loss

Mitochondria are membrane-bound organelles found within most eukaryotic cells and are responsible for generating adenosine tri-phosphate (ATP), a molecule that is used as a source of chemical energy within the body (Scheffler, 2008; Copeland, 2002). The number of mitochondria contained within a cell depends on the organism as well as the tissue type and can range from a couple of hundred to a few thousand. Cells within tissue that requires a lot of energy to function, like muscle tissue and the inner ear, often contain more mitochondria (Scheffler, 2008; Copeland, 2002).

One important distinction that separates mitochondria from other organelles within the cell is that mitochondria have their own genome made up of several copies of a double-stranded chromosome that takes the shape of a ring (rather than a helix, as found in nuclear DNA) with its own genetic code (Barrell *et al.*, 1979). The human mitochondrial genome consists of 16,569 base pairs (Anderson *et al.*, 1981, GenBank,) that make up 37 genes: 13 code for the proteins that interact with nuclear proteins to carry out oxidative phosphorylation, 22 code for the transfer RNA required for the mitochondrial protein-synthesizing system, and 2 code for the large and small subunits of

ribosomal RNA (taken from www.ncbi.nlm.nih.gov/genbank/ on November 29, 2011, Fischel-Ghodsian, N., 2002).

The mitochondrial genome is maternally inherited. While both egg and sperm contain mitochondria, the mitochondria from the sperm are lost during early embryogenesis (Manfredi *et al.*, 1997). Therefore, a mother may pass on any mutations in her mitochondrial genome to all of her children. Determining whether or not the mutation will be passed on depends on the number of mitochondrial chromosomes or mitochondria that contain the mutation. For example, some mitochondrial mutations are heteroplasmic, that is, they are only present within some mitochondria out of all mitochondria that are contained in the cell. Other mitochondrial mutations are homoplasmic, meaning the mutation is present in all of the mitochondria (Wallace, 1992). In the case of a heteroplasmic mutation the proportion of wild type or mutant genes that are passed on is random. It is possible that a mother will pass on only those mitochondria that do not carry the mutation and therefore no phenotype will appear (Fischel-Ghodsian, N., 2002). Mitochondrial mutations are difficult to pinpoint because there are numerous DNA molecules within a single mitochondrion and there are numerous mitochondria per cell.

In the early 1990s, it was discovered that mutations in mitochondrial genes were linked to hereditary hearing loss (Hu *et al.*, 1991; Jaber *et al.*, 1992; Prezant *et al.*, 1993). Hu *et al.* (1991) analyzed 36 pedigrees with a positive history of aminoglycoside induced hearing loss and in 22 of the pedigrees where the pattern of inheritance could be ascertained they found that transmission of the predisposition to develop a hearing

impairment after aminoglycoside exposure was exclusively through females. They considered two explanations for this type of transmission: the first was that it could be an X-linked predisposition; the second was that it could be caused by mitochondrial inheritance. They ruled out X-linked transmission as there was equal inheritance in females and males and the affected males did not pass the predisposition on to their daughters or the sons of their daughters. Because they could exclude X-linked inheritance this left them with the conclusion that aminoglycoside induced hearing loss was a mitochondrially inherited disorder. Hu *et al.* were the first group to come to this conclusion, and at the time there were only a few rare diseases shown to be mitochondrially inherited. Following this conclusion, the next step Prezant *et al.* (1993) took was to look for mutations in the human ribosomal RNA (rRNA) genes, which are found in mitochondrial DNA, in those families. The investigators chose this area to begin their mutation search because they knew that the rRNA of bacteria was the target of these antibiotics. As well, they decided to sequence the entire mitochondrial genome in a large Arab-Israeli family. Each of the four families had a substitution of a guanine for an adenine at position 1555, within the 12S rRNA gene. This was the first case of a mitochondrial mutation being associated with non-syndromic hearing loss. It was discovered that the A1555G mutation in the mitochondrial gene *MTRNR1*was one of the most frequent causes of hearing loss after mutations in *Cx26*, and *SLC26A4* (Solute carrier family 26 member 4, the gene mutated in Pendred syndrome – deafness with thyroid enlargement, and in DFNB4 with enlarged vestibular aqueduct; taken from http://omim.org/ on February 13, 2012; Guo *et al.*, 2008).

Since the early 90s, many families have had their hearing loss explained by mutations in mitochondrial genes. As well, in the case of aminoglycoside-induced hearing loss, it is possible to prevent the development of a hearing impairment by avoiding aminoglycoside antibiotic therapy in individuals with a family history of aminoglycoside induced hearing loss (Hu *et al.*, 1991; Selimoglu, 2007). There are now two mitochondrial genes associated with NSHL (Retrieved from http://hereditaryhearingloss.org/ on November 25, 2011). Neither of these genes are protein coding; one, *MTRNR1*, codes for 12S ribosomal RNA, the small subunit of mitochondrial ribosomes that binds with the large subunit, 16S, to carry out protein synthesis within the mitochondria. The second, *MTTS1*, codes for the serine (UCN) transfer RNA, the RNA that carries serine to the polypeptide chain during protein synthesis when it is coded for by the triplet AGN (OMIM, http://omim.org/). There are three mutations in *MTRNR1* that are associated with both aminoglycoside-induced hearing loss and NSHL that is not triggered by aminoglycoside exposure. There are four mutations reported to cause nonsyndromic hearing loss in *MTTS1*, however two of these mutations are also associated with another disorder. The c.7445A>G mutation is associated with palmoplantar keratoderma (PPK) and the c.7472insC mutation is associated with neurological dysfunction, including ataxia, dysarthria and myoclonus (Hereditary Hearing Loss Homepage http://hereditaryhearingloss.org/).

The first of these mitochondrial mutations to be associated with hearing loss was discovered in an Israeli-Arab pedigree with hearing loss inherited through the maternal line (Jaber *et al.*, 1992; Prezant *et al.*, 1993). The hearing loss was progressive and

usually presented in childhood. It was Prezant *et al.* in 1993 that sequenced the entire mitochondrial genome of family members from the Israeli-Arab pedigree and also from three unrelated patients with familial aminoglycoside-induced deafness. They found a point mutation at position 1555 that changed an adenosine to a guanine in the gene that codes for the 12S ribosomal RNA (rRNA). This mutation was shared amongst all four families. However, Prezant *et al.* were not the first to suggest that familial aminoglycoside-induced deafness was caused by a defect in the mitochondrial DNA. In 1989, Higashi looked at 28 Japanese families to determine whether males or females passed on the trait of susceptibility of streptomycin deafness. After examining each pedigree he concluded that it was in fact transmitted through only females. Until this discovery, the susceptibility of the cochlea to streptomycins was assumed to be autosomal dominant (Higashi, 1989). Higashi (1989) also suggested that because familial hearing loss induced by streptomycin exposure could not be explained by ordinary Mendelian inheritance, then ototoxicity caused by streptomycin intake somehow disrupted ATP production in the mitochondria of the hair cells, and that might be caused by changes in the mitochondrial DNA.

Once the A1555G mutation had been discovered in aminoglycoside induced deafness it was also found to cause nonsyndromic hearing loss worldwide, in Arab-Israeli, Japanese, Mongolian, Zairean, Spanish, Chinese, Turkish, and Balinese families (Usami *et al.*, 1997; Estivill *et al.*, 1998; Kupka *et al.*, 2002; Abreu-Silva *et al.*, 2006; Kokotas *et al.*, 2009). In fact mutations in connexin 26, *SLC26A4*, and mtDNA A1555G are the most prevalent causes of deafness worldwide (Guo, 2008). This means that

screening for the mtDNA A1555G mutation should be included when looking for the underlying genetic cause of hearing loss in a family, especially if the inheritance appears to be maternally transmitted or hearing loss occurred after aminoglycoside use.

The first mutation identified in *MTTS1* changed an adenosine at position 7445 to a guanine. It was found by Reid *et al.* in 1994 in all affected members of a family with 13 members displaying sensorineural NSHL. The second *MTTS1* mutation was an insertion of a cytosine at position 7472, found by Tiranti *et al.* in 1995 after sequence analysis of the tRNA gene regions of mitochondria in affected individuals from a large Sicilian kindred. This mutation causes nonsyndromic hearing loss as well as hearing loss accompanied by ataxia and myoclonus (Tiranti, 1999). The third *MTTS1* mutation found to cause hearing loss was the change of a thymine to cytosine at position 7511 (7511 T>C), a mutation discovered by Sue *et al.* in 1999 in 36 affected members of a large African American family when they sequenced the entire mitochondrial genome in search of mutations because of the maternal transmission of hearing loss. This mutation, which disrupts a highly conserved site in the mitochondrial DNA, was determined by Sue *et al.* (1999) to be pathogenic. The fourth *MTTS1* mutation found thus far is a change from thymine to cytosine at position 7510 (7510 T>C), and was identified in a Caucasian family by Hutchin *et al.* in 2000. Hearing loss in this family appeared to be transmitted through the maternal line so they looked for mutations in the mitochondrial DNA. First they screened for other known mitochondrial DNA mutations, but all were absent. They then found the T to C transition at base pair position 7510. This novel thymine to cytosine transition at position 7510 was found in all affected family members and was not

found in 141 Caucasian controls. As well the thymine at position 7510 is highly conserved in a wide range of species (Hutchin, 2000). These mutations in the *MTTS1* gene should also be included when screening for genetic hearing impairment, especially in families where hearing loss is transmitted through the maternal line.

1.3 Summary

Hearing loss is a very debilitating disorder that causes many problems in a developing child. If hearing loss is pre-lingual, a child will have difficulty learning to speak, as children acquire this ability by imitating sounds they hear around them, such as the voices of parents and siblings. The longer hearing loss remains undetected the greater the likelihood for permanent impairments of speech and language that are necessary for social development and the ability to take part in a standard education system (NIDCD website). Through the study of hearing loss genetics, the underlying causes of inherited hearing impairment can be revealed, improving the lives of those affected by the disorder.

Co-authorship statement

I, Jessica Squires, hold a principal authorship status for all of the manuscript chapters in my thesis. However, the manuscript is co-authored by my supervisor, Dr. Terry-Lynn Young, who helped design my project and research proposal, and also assisted in data analysis and review of my manuscript. Co-authorship is also given to PhD students: Dr. Lance Doucette and Dr. Nelly Abdelfatah who helped with review of my manuscript and the Young Lab Research Assistants: Mr. Dante Galutira, Mr. Jim Houston, and Mrs. Tammy Benteau who helped with data analysis and manuscript review.

2.Materials and Methods

2.1 Subject Recruitment

Probands with hearing impairment, along with their relatives, were recruited from across Newfoundland and Labrador. This study focused on predominantly probands with profound hearing loss as children. Probands and their blood relatives were recruited to the study through various poster drives and through the Provincial Medical Genetics Program of Eastern Health, St. John's. Family history, informed consent, and permission to access medical records and audiograms were obtained as per approved protocol #01.186 (Human Research Ethics Board, St John's, NL, Canada). The team also collected information on the geographical location of family founders. The patients and/or their parents/guardians completed a hearing loss questionnaire (Appendix 2), and a blood sample for DNA isolation was obtained from all available family members. Probands with hearing impairment due to known environmental factors such as infection and acoustic trauma were excluded from further study.

2.2 Overall Design of Candidate Gene Approach

A step-wise, targeted screening approach was used to look for mutations across three categories of deafness genes: (1) genes with mutations previously identified in hearing impaired probands with Newfoundland ancestry; (2) genes frequently mutated in Caucasians with hearing loss, and (3) genes that cause specific, recognizable patterns of hearing loss as seen on an audiogram. When a pathogenic mutation was identified in a

proband, I confirmed, where possible, which side of the family the mutation came from by genotyping DNA from the parent(s) and other available blood relatives.

(1) Screening genes identified in hearing impaired probands from Newfoundland

Preceding this study, working as a Research Assistant in Dr. Young's laboratory, I screened 77 probands for mutations in genes previously found to cause hearing loss in the Newfoundland population; namely *WFS1, TMPRSS3* and *PCDH15* that our lab previously identified in deaf probands from Newfoundland (Young *et al.*, 2001; Ahmed *et al.*, 2004; Doucette *et al.*, 2009) and for common mutations causing hearing loss in *Cx26* and *Cx30*. The analysis of this data is part of my thesis project. In order to detect potential recurrent mutations due to founder effects in the Newfoundland population and potentially (and efficiently) solve one or more probands for my thesis project I also sequenced genomic DNA of 33 newly ascertained probands for mutations in *WFS1, TMPRSS3* and *PCDH15*.

(2) Screening Genes identified in Caucasians with Hearing Loss

In order to detect common mutations in the *Cx26* and *Cx30,* two different protocols were required. To detect the common point mutations in Cx26, full, bidirectional Sanger sequencing of *Cx26* was done on DNA from the newly ascertained probands (n=33). However, Sanger sequencing is not sufficient to detect the common mutation in Cx30, as it is not a point mutation but a large deletion. Therefore, a PCR-based method, first described by del Castillo *et al*. (2002), was used to detect the common 342 kb deletion (del13S1830) in *Cx30* in the newly ascertained probands (n=33).

The next step was to look for common mutations that occur in the mitochondrial genome that can also lead to hearing loss. To do this, mitochondrial DNA from all unsolved probands (n=101/110) was screened for mutations in the mitochondrial genes *MTRNR1* and *MTTS1* using full, bidirectional Sanger sequencing.

(3) Screening Genes Causing Recognizable Audiogram Patterns (Audioprofiles)

In some cases, the underlying causative gene can be suggested by patient audioprofiles, or pattern of loss as seen on an audiogram. For the majority of probands in this study, at least one audiogram was available and the audiograms were analyzed and grouped into four categories (low-frequency loss, mid-frequency loss, high-frequency loss and flat loss; Figure 2.2).

2.3 Techniques for Mutation Detection

1. Extraction of Genomic DNA

Genomic DNA was extracted from the blood of affected probands and their relatives and archived and stored at 4°C. For the 33 new recruits, I extracted genomic DNA from peripheral blood (leukocyte fraction) according to a standard salting out protocol (Appendix 3).

2. Amplification of Targeted Sequences by Polymerase Chain Reaction (PCR)

PCR reactions contained 1X Kapa Taq Buffer A with 1.5 mM $MgCl_2$ (Kapa Biosystems, Woburn, MA), 0.9 M betaine (Sigma, Saint Louis, MO) 0.2 mM dNTPs (Applied Biosystems, Warrington, UK), 0.4 uM of each primer (IDT, San Diego, CA), 0.4U of Kapa Taq, and 10 ng of purified DNA in a total volume of 20 µL. This master

mix was used for all genes with the exception of *Cx26*. The master mix for *Cx26* did not contain betaine. PCR was performed on a GeneAmp PCR System 9700 thermocycler (Applied Biosystems, Foster City, CA) with cycling conditions of [94^{o}C/ 5 min, (94^{o}C/ 30 s, 64^{0}C – 2.0^{0}C per cycle/30 s, 72^{0}C/ 30 s) x 5 cycles, (94^{o}C/ 30 s, 54^{o}C/ 30 s, 72^{0}C/ 30 s) x 30 cycles, 72^{0}C/ 7 min]. The primers and amplification conditions for each gene are provided in Appendix I. The PCR products were stained and run on a 1% agarose gel (0.5g agarose/ 50mL 1X TBE + 2.5µL SYBR safe/ 50mL of 1X TBE) using gel electrophoresis, and viewed with UV light using the Kodak Molecular Imaging system. This allowed the product size of the PCR products to be compared to a standard DNA ladder in order to be sure the anticipated size of DNA segment (PCR product) was obtained.

3. Preparation for Sanger Sequencing using ABI Cycle Sequencer

Samples were prepared according to standard lab protocols: 300µL of Sephacryl 300 was added to each well of a 96 well, 0.45 µm MultiScreen filter plate and centrifuged for 5 minutes at 1800 x g. The flow-through was then discarded. Next, the PCR products were added to the wells of the MultiScreen plate. A 96 well PCR catch plate was placed beneath it and the plates were centifuged at 1800 x g for 5 minutes. This time the flow-through was retained in the PCR plate and the purified PCR product was prepared for sequencing.

A sequencing master mix was prepared to contain the following ingredients: Sequencing Mix (0.5µL), 5X Sequencing Buffer (2.0µL), Primer (either forward or reverse) at 1.6 mM (2.0µL), and dH_2O (14.5µL). This mixture was the same for all genes and yielded

19.0µL volume per well of a sequencing plate. 1 µL per well of purified PCR product was added to the master mix and then centrifuged briefly and placed on a thermocycler for cycle sequencing with cycling conditions of [(96^{o}C/ 1 min, 96^{o}C/ 10 s, 50^{0}C/5 s, 60^{o}C/4 min) x 24 cycles, 4^{0}C hold].

The next step was ethanol precipitation of the PCR product. 5.0 µL of EDTA (125mM) followed by 65 µL of ethanol (95%) were added to each sample in the sequencing plate. The plate was then covered and placed at room temperature in the dark for 30 minutes to 24 hours to precipitate the DNA. Next, the plate was centrifuged for 30 minutes at 3000 rpm. The plate was then inverted to decant the ethanol and while still inverted was centrifuged briefly at 200rpm to further decant the ethanol. 150.0 µL of ethanol (70%) was then placed in each well. The plate was centrifuged for 15 minutes at 3000 rpm and inverted to decant the ethanol, and then the inverted plate was briefly centrifuged at 200 rpm to remove residual ethanol. The plate was left to dry in the dark for 10-15 minutes to evaporate any remaining ethanol and dry the DNA pellets. The DNA was dissolved in 15.0 µL of HDF (High-Dye Formamide), centrifuged briefly, and placed on the thermocycler for denaturing (2 mins at 95ºC).

Automated sequencing was performed on the Applied Biosystems 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA). The raw sequencing data was inspected to check the quality and then analyzed with Mutation Surveyor DNA Variant Analysis Software V4.0.6 (State College, PA), which identifies sequence variants in the sample by comparing the sample sequence to a reference sequence.

4. Connexin 30 Primers and Conditions for Detecting 342kb Deletion

The *Cx30* gene contains a 342kb deletion that has been associated with hereditary hearing loss (del Castillo *et al*., 2002). Detection of this deletion is different from the detection of other, mainly point mutations in this study. Rather than using Sanger sequencing, the presence of a deletion is detected by analyzing band sizes of the PCR products on an agarose gel. The forward and reverse strands of a segment of *Cx30* were amplified to detect the large 342kb deletion (del13S1830) (del Castillo *et al.*, 2002). Two primer sets were used according to the del Castillo protocol; the first primer set, called "Connexin 30 c" (Appendix 1, Table 1), will amplify a 651 bp segment of the intact *Cx30* gene (when there is no deletion). If the 342kb deletion mutation is present, the reverse primer cannot bind, resulting in no PCR product. The second primer set "Connexin 30 d" has a forward primer and reverse primer that anneal at opposite ends of the gene (Appendix 1, Table 1). In a normal gene sequence, these primers lie too far apart to produce a PCR product. However, the large deletion mutation in *Cx30* removes 342kb of sequence and by doing so, brings the forward and reverse primers in close enough proximity to each other to produce a PCR product (405 bps). This approach provides a positive test for the detection of the deleted allele.

In order to carry out the PCR test for the Cx30 deletion mutation, the four primers were combined (0.2µM each) into one PCR mixture (0.4 µL each). The other reagents in the PCR mixture include: 10x Buffer with Mg^{2+} (2.0µL), dNTPs (2mM; 2.0µL), Taq Polymerase (0.08µL), betaine (4.0µL), and dH2O (9.32µL). This 19.0 µL total volume was multiplied according to the number of samples being tested. The mixture was

aliquoted into wells on a PCR plate along with 1.0 μL of DNA and placed in a thermocycler for amplification (see Appendix 1,Table 1 for cycling conditions).

The sizes of the PCR products were determined after they were separated by electrophoresis through a 1% agarose gel (0.5g agarose/ 50mL 1X TBE + 2.5μL SYBR safe/ 50mL of 1X TBE). The genotype of each proband at the *Cx30* locus was easy to determine with this PCR-based test. The normal sequence produces a PCR product 651 bps in size. A sequence with the deletion produces a PCR product of 405 bps (del Castillo *et al.*, 2002). A proband with two copies of the normal (undeleted) *Cx30* gene would yield a single 651 bp band; a proband heterozygous for the deletion would yield two bands (one 651 bp and one 405 bp); an individual homozygous for the deletion mutation would yield a single 405bp band.

5. Detection of a Possible Mitochondrial Deletion

Because mitochondrial traits are passed down maternally and can appear to be transmitted in a dominant or recessive manner, at this stage of the targeted screening protocol, it was prudent to screen all unsolved probands for potential mutations. Three sequencing primer pairs were designed to overlap each other in order to fully cover the *MTRNR1* gene, and one primer pair was designed to cover the *MTTS1* gene (Appendix I). For example, the first primer pair for *MTRNR1* covered positions 133 to 941, the second primer pair covered positions 614 to 1463, and the third pair covered positions 1273 to 1771. These position numbers indicate the position within the circular mitochondrial genome. The PCR products, using primers 133F-941R, were examined by gel

electrophoresis to check for the correct product size. For a normal MT sequence, the expected band size is approximately 800 bps.

5. Verification of Pathogenicity

There are several steps to help verify if a variant is pathogenic, that is, causative for deafness. Mutations were deemed pathogenic if a literature search of high quality publications revealed strong evidence that the mutation was pathogenic. For novel variants, they were also tested for co-segregation with hearing loss in the proband's extended family. The published frequency of the mutation was determined from the SNP database, and by genotyping population controls of unaffected individuals with Newfoundland ancestry. Furthermore, the predicted effect on the protein was estimated using five bioinformatics programs including SIFT, Polyphen, SNPs3D, PMut, and Weblogo. A summary of the experimental design is provided in figure 2.1.

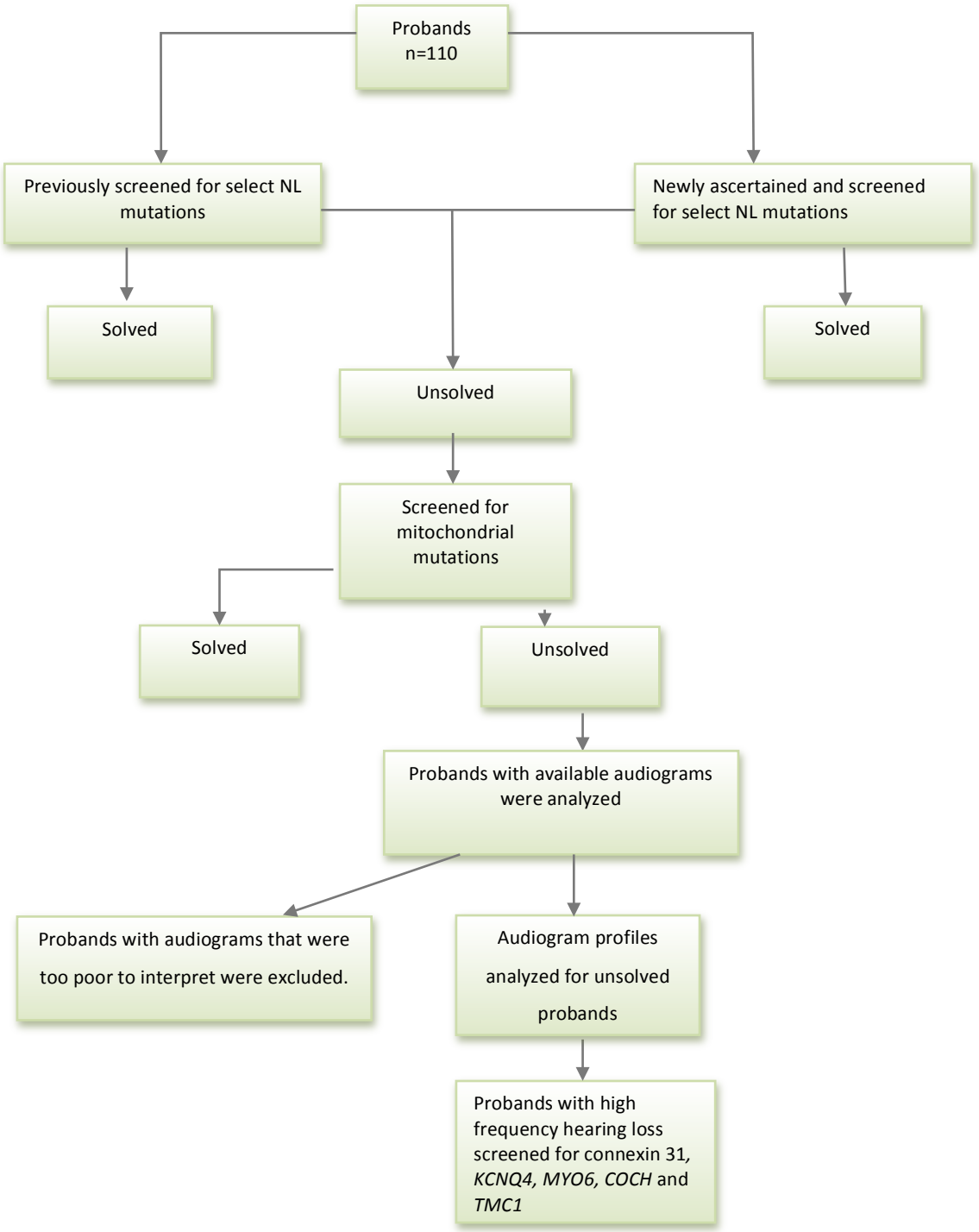


Figure 2.1. Flowchart outlining the experimental design of this project.

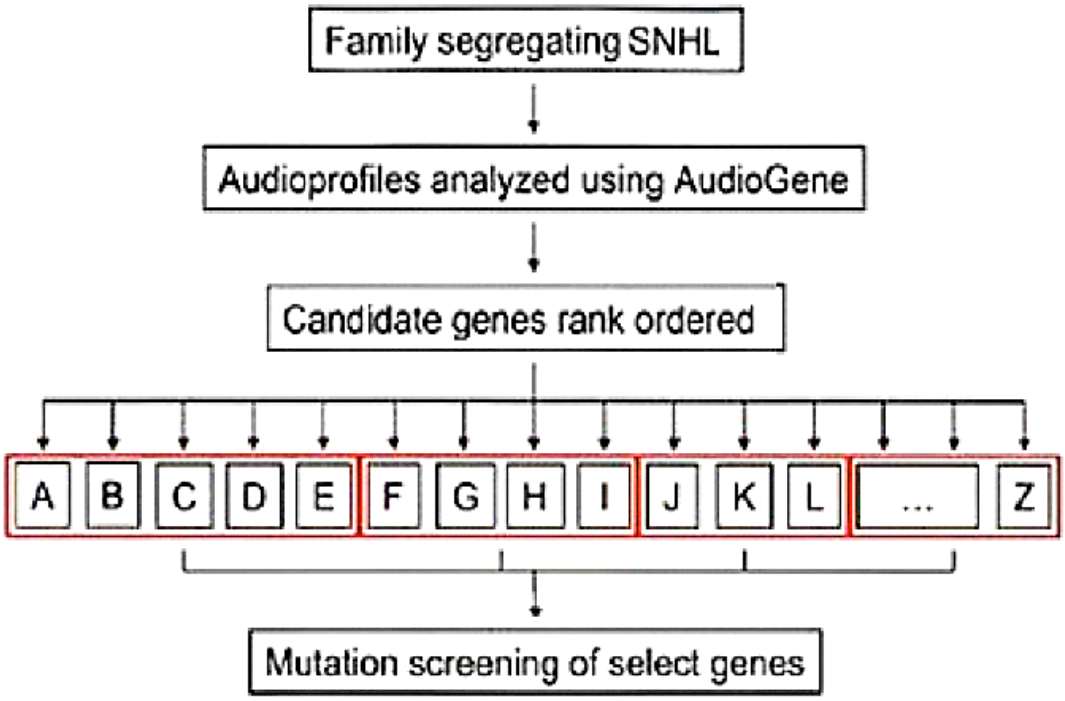


Figure 2.2. Steps of the process of AudioGene candidate gene determination and analysis (SNHL = Sensorineural Hearing Loss; Adapted from audiogene.eng.uiowa.edu)

3. Results

A candidate gene approach, using a step-wise strategy, was developed to identify the genetic cause of hearing loss in 110 probands. The candidate gene approach began with 33 newly recruited probands. These new recruits to the study were first screened for deafness mutations identified in the Newfoundland population by our lab. Following this screening, all unsolved probands were combined with the 69 unsolved probands from our previous studies for a total of 101 unsolved probands (Fig. 3.1).

3.1 Screening for deafness mutations previously identified in Newfoundland

Direct, bidirectional Sanger sequencing of the amplicons covering pathogenic mutations in three genes, *WFS1* (8 exons), *TMPRSS3* (13 exons), and *PCDH15* (35 exons) all yielded high quality sequences for each of the 33 samples (Fig. 3.2). However, none of the 33 probands were heterozygous or homozygous for the 3 known deafness mutations. In other words, all samples yielded a normal sequence indicating that hearing loss in the probands is not due to deafness mutations previously identified in patients with Newfoundland ancestry.

3.2 Screening genes identified in Caucasians with hearing loss

The next step in the strategy was to carry out Sanger sequencing on exons covering recurrent mutations in populations with similar ancestry to the Newfoundland population. For this step, I screened the NL probands for gene mutations known to frequently underlie hearing loss in patients of Northern European decent.

3.2.1 Probands with variants in connexin 26 and connexin 30

For the 33 newly ascertained probands, full gene sequencing of *Cx26* was carried out. As *Cx26* has only two exons, one of which is coding, screening this entire gene was straightforward. Screening of both exons of *Cx26* across the 33 samples yielded 30 normal sequences and 3 mutated sequences. I detected 2 variants, a single base pair deletion and common pathogenic mutation causing nonsyndromic hearing loss (Fig. 3.3). The deletion of one guanine in a chain of six guanines in Cx26 causes a reading frameshift in the sequence. Figure 3.3 shows the typical pattern that is seen on an electropherogram in a person carrying one copy of the 35delG mutation.

The second variant detected in the *Cx26* gene was a missense mutation, c.250A>AG, p.F83L, determined to be nonpathogenic following bioinformatics analyses including SIFT, Polyphen, SNPs3D, PMut, and Weblogo. A literature search also revealed that the F83L variant was considered to be nonpathogenic in other studies. (Retrieved from http://davinci.crg.es/deafness/index.php on October 13, 2010).

Screening for the Cx30 deletion was not done by Sanger sequencing, but rather by examining PCR products (del Castillo *et al.*, 2002). The deletion spans 342 kb and is therefore too large to be detected using Sanger sequencing. Instead, the deletion can be detected using a set of four primers designed by del Castillo *et al.* (2002) to amplify the truncated construct that results from the deletion. The first set of primers will only amplify a 651bp region in the absence of the deletion, this is because the reverse primer is designed to anneal to the new sequence that is created in the region of the repaired

chromosome. The second set of primers will amplify a 405bp region when the deletion is present; otherwise the primers lay too far apart on chromosome 13 to allow for amplification. Both amplicons can be distinctly identified on an agarose gel (Fig. 3.4). Although it took several attempts to get all samples to amplify, eventually all 33 samples worked. Of the 33 samples, 1 sample was positive for the Δ(*GJB6*-D13S1830) deletion.

The *Cx26* mutation, c.35delG, and the Δ(*GJB6*-D13S1830) deletion in *Cx30*, were both identified in the proband from Family 2155 (Fig.3.5), a singleton family. Together this digenic inheritance of recessive mutations cause hearing loss, that is, the proband is heterozygous for the c.35delG mutation in *Cx26* (Fig.3.3) and heterozygous for the Δ(*GJB6*-D13S1830) deletion in *Cx30* (Fig.3.4). According to the family history data, the parents are unaffected. Therefore, the proband may have a recessive form of hearing loss, or alternatively, the hearing loss is due to two *de novo* mutations, or one segregating mutation and one *de novo*, although both of these latter possibilities are less likely. Because there was no DNA available for the parents, I was not able to determine which parent carried which mutation, or if either of the mutations were in fact, *de novo* mutations.

The proband from family 2197 (Fig.3.6) was found to be heterozygous for the c.35delG mutation in *Cx26* (Fig.3.7). The proband had an affected father as well as two affected uncles. The paternal grandmother was also affected, suggesting that hearing loss was segregating as a dominant trait on the paternal side. However a second mutation was not found in *Cx26* or *Cx30*, and there is no further information or DNA available for

other members of the family. Although there are mutations in *Cx26* that cause dominant nonsyndromic hearing loss, 35delG is not one of them (Retrieved from http://davinci.crg.es/deafness/index.php on October 9, 2014).

Family 2091 has three affected members, the proband (III-11), a paternal cousin (III-1), and paternal grandfather (I-1), with no evidence of vertical transmission, making the inheritance of hearing loss in the proband appear to be recessive (Fig.3.8). DNA was only available for the proband who was identified to have a variant in *Cx26*, a single base pair substitution leading to a missense mutation (c.250A>AG) causing the change of a phenylalanine to a leucine (p.F83L) (Fig. 3.9). Using SIFT, Polyphen, SNPs3D, PMut, and Weblogo, this polymorphism was predicted to be benign (Table 1, Fig 3.10).

3.2.2 Probands with variants in *MTRNR1* and *MTTS1*

The A1555G mutation in mitochondrial gene *MTRNR1* is one of the most frequent causes of hearing loss after mutations in *Cx26* (Guo *et al.*, 2008), therefore it was important to include screening of mitochondrial DNA in this study. In addition, I decided to screen the *MTTS1* gene because it is the only other mitochondrial gene in which mutations are known to cause nonsyndromic hearing loss (Retrieved from hereditaryhearingloss.org on September 1, 2014). Screening of mitochondrial genes *MTRNR1* and *MTTS1* was carried out on 101 probands, in two rounds. In the first round, twenty probands were screened to perfect the mitochondrial screening process. Full gene sequencing was completed for both genes, and six polymorphisms (Guaran *et al*., 2013;

Li *et al*., 2004) (Table 2) were found in *MTRNR1*, however no mutations were identified in *MTTS1*.

The G951 variant, the change from a guanine to an adenosine at position 951, was identified in this group in one proband, family 2167 (Figs.3.11 and 3.12). This variant has been reported in literature, however its current status is nonpathogenic (Elstner *et al.*, 2008).

There were 81/101 probands included in the second stage of screening (Fig.2.1), which began when mitochondrial sequencing was perfected and included the remaining unsolved probands. In addition to the six polymorphisms identified in the 20 probands screened in the first round, two polymorphisms were found (G709A and T1243C) in *MTRNR1.* Two probands, one from each of family 2112 and family 2144, (Figs. 3.13 and 3.14), were found to carry the homoplasmic A1555G mutation (Figs.3.15 and 3.16), known to cause hearing loss following exposure to aminoglycosides (Guan, 2011).

3.2.3 Challenges with Mitochondrial Screening

Analysis of the first section of *MTRNR1* indicated that the proband from family 2072, as well as the proband's mother (Fig.3.17), had a lower molecular weight amplicon than expected, as seen on the agarose gel (Fig.3.18). This could be interpreted as a deletion; however there was no clear evidence of a deletion, i.e., a frameshift in the sequence, upon sequencing the truncated PCR product. For reasons unknown, the quality of the sequencing was poor up to position 580 in the mitochondrial genome. Upon examining these results with Mutation Surveyor®, it was noticed that the sequence of the

MTRNR1 forward primer was showing up in the reverse sequence, however it was 349bps downstream from where this sequence should be, according to the reference sequence. The forward sequencing primer was designed to sequence position 133 of the mitochondrial DNA onward (Fig. 3.19).

A second attempt was made to sequence across this region in the probands' MT genome. In order to do this, I designed a new primer set. This approach was successful, and after sequencing with the new primers, I discovered that there were two SNPs (C150T and T152C) where the 3' end of the original forward primer was expected to anneal at position 133, which explains why the sequence was initially poor and why the amplicon for these individuals was much smaller.

Once the SNPs, C150T and T152C, were discovered in the proband from family 2072, the agarose gel for the original *MTRNR1* primer set was re-analyzed and it was found that a second proband, from family 2010 (Fig.3.20), had the same unusual lower molecular weight amplicon. I subsequently screened this second proband, along with five of her relatives, for the two control region SNPs (C150T and T152C). Because mtDNA is only passed down through the maternal line, as expected, all maternal relatives of the proband had these SNPs (C150T and T152C), and a paternal uncle had a different haplotype. These results help to validate the utility of my newly designed MT sequencing primers and confirm my suspicions regarding the unusual findings.

Following the successful re-sequencing of family 2010, all probands in the study were screened for these interesting SNPs and a table was created in order to compare the

mitochondrial haplotypes of all deafness probands (Table 3). From this exercise, I could see that only Families 2072 and 2010 carried C150T and T152C in their MT genomes. I then reasoned that if these families are related, it is also reasonable that hearing loss might be due to the same underlying genetic etiology. Therefore, I compared the audiograms of affected members across the two families (persons III-1, II-4 from family 2072; person XI-7 from family 2010; Fig.3.21) and submitted the audiograms to AudioGene (Retrieved from http://audiogene.eng.uiowa.edu/ on May 8, 2011). The first gene predicted to underlie the pattern of hearing loss was *KCNQ4*. I sequenced the entire coding sequence and exon/intron boundaries of *KCNQ4* in these individuals, however no variants were found. In a similar fashion, other families with similar mitochondrial haplotypes were considered together. Table 3 depicts probands who have matching haplotypes. Potentially, the probands with matching haplotypes are linked on the maternal side of the family. The blank spaces in Table 3 indicate where multiple sequencing attempts failed to identify the correct nitrogenous base.

3.3 Screening genes causing recognizable audiogram patterns (audioprofiles)

From the unsolved probands (n=99), audiograms from 55 probands were compared and grouped into four hearing loss categories: low frequency loss, mid frequency loss, high frequency loss, and all frequency loss - flat line (Table 4). Audiograms were submitted to AudioGene for a potential match to a candidate gene(s). In the high frequency category, which included 29 families, five candidate genes (*Cx31*, *KCNQ4*, *MYO6*, *COCH*, and *TMC1)* were identified as potential targeted genes by AudioGene. In the mid frequency category, only one gene (*TECTA)* was predicted. For

the low and flat categories, three candidate genes (*WFS1*, *POU4F3*, and *DIAPH1)* were indicated.

Bidirectional Sanger sequencing of the 29 probands with high frequency hearing loss was done for *Cx31*, *KCNQ4*, *MYO6*, *COCH*, and *TMC1* and revealed a total of five variants. Two variants were identified in *Cx31*: c.109G>A, p.V37M and c.94C>T, p.R32W, three were identified in *TMC1*: c.421C>T, p. R141W; c.545G>A, p.G182D; and c.1763+3A>G (Table 5).

Of the two variants found in *Cx31*, the c.94C>T: p.R32W has a frequency of 0.015 in the SNP database (Retrieved from http://www.ncbi.nlm.nih.gov/ projects/SNP/ on July 15, 2014). This mutation was heterozygous in the proband from each of six families: family 2155 (Fig. 3.5), family 2176 (Fig 3.11), family 2078 (Fig. 3.22), family 2075 (Fig. 3.23) and family 2097 (Fig. 3.24), family 2156 (Fig 3.25). The proband from family 2083 carried a single copy of the c.109G>A:p.V37M variant, a novel mutation (Fig. 3.26). The proband from family 2083 is also heterozygous for the *Cx30* deletion Δ(*GJB6-D*13*S1830*). Screening of the available family members of these probands (families 2078, Fig 3.27; 2075, Fig 3.28; 2097, Fig. 3.29; and 2083, Fig. 3.30) revealed that the all of the mutations segregated with hearing loss.

Heterozygous mutations in *TMC1* were detected in 3 probands with high frequency hearing loss. The R141W missense mutation was found in the proband from family 2010 (who also has mutations in the mitochondrial DNA as previously mentioned) (Fig.3.20), the G182D in the proband from family 2124 (Fig. 3.31), and c.1763+3A>G in the proband from family 2065 (Fig. 3.32).

Following the successful screening of high frequency hearing loss families, the rest of the unsolved deafness families were also screened for these newly found mutations, and subsequently, three more probands were identified with the heterozygous c.421 C>T, p. R141W mutation in *TMC1*: Family 2146 (III-6; Fig.3.33), Family 2177 (IV-2; Fig. 3.34) and Family 2092 (III-4; Fig. 3.35). Cascade sequencing revealed 3 other family members in 2010 (Fig. 3.20) with the heterozygous R141W mutation and 3 other family members in 2092 (Fig. 3.35) with the heterozygous R141W mutation.

Population screening for the three *TMC1* mutations produced the following results: c.421 C>T p.R141W (dbSNP frequency of 0.001) was found in 2 out of 134 or 1.5% of alleles in ethnically matched population controls, which falls slightly above the boundary for being considered a rare variant (<1% population frequency). The variant c.545 G>A p.G182D was not found in dbSNP and was absent in 94 alleles of ethnically matched population controls, leading to the belief that it could be a rare variant and c.1763+3A>G was not present in dbSNP was absent in 102 alleles in ethnically matched population controls, again, evidence that it could be a rare variant. All findings are indications that the detected variants are likely pathogenic.

3.5 Summary of Results

Several significant findings came out of this research project. First of all, it was discovered that mutations previously identified in the NL population were not found in any of the probands included in this study. This would lead me to believe that these NL mutations are not founder mutations. If they were, I would expect them to be identified in

a higher number of hearing impaired probands in the population. Instead these mutations seem to be isolated to distinct families.

Secondly, both the 35delG mutation in Connexin 26 and the large deletion, del13S1830, in Connexin 30 were found in two of the probands. One proband was found to be heterozygous for both mutations (35delG and del13S1830), known as digenic inheritance, causing hearing loss. One proband was a carrier for the 35delG mutation in connexin 26. These mutations are the most common cause of early onset hereditary hearing loss in Northern European populations. These findings would be expected as many families in the current population have Northern European heritage.

In addition to this, several variants were identified following audiogram analysis and candidate gene identification using AudioGene. Two variants were found in Connexin 31, one known variant (R32W), and one novel variant (V37M) which appeared to segregate with hearing loss in four families. Three novel variants were found in *TMC1* (R141W, G182D, and c.1763+3A>G), these variants were not found in high frequency in ethnically matched population controls.

This study also identified two individuals who had the A1555G mutation in the *MTTS1* gene, a mutation causing hearing loss or making the individual more susceptible to developing hearing loss following the administration of aminoglycosides. The screening of the mitochondrial genome also lead to the discovery of possible relationships among families by comparing their mitochondrial haplotypes.

Overall, a total of 10 significant variants were found in 18 probands, 7 of these variants require further discussion and research and 3 are known to be pathogenic and cause hearing loss. A detailed summary of the gene variants found in this study is shown in Table 6.

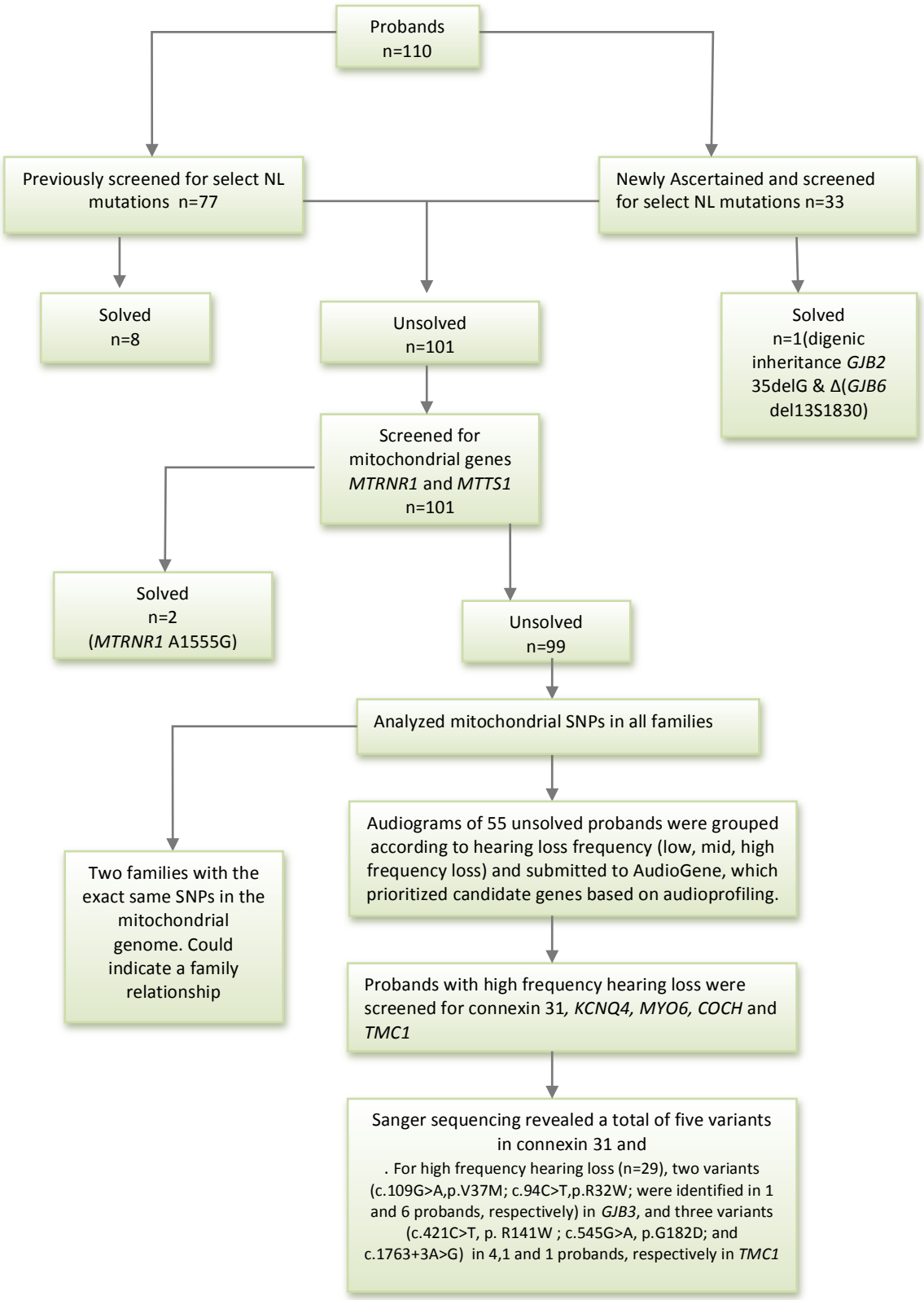
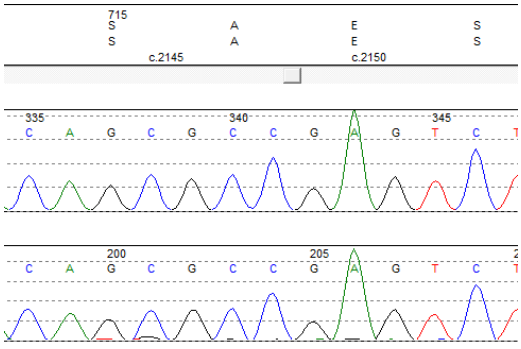
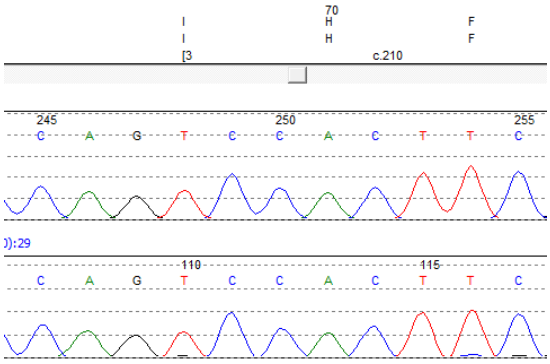


Figure 3.1. Flowchart outlining the methods followed with results included.

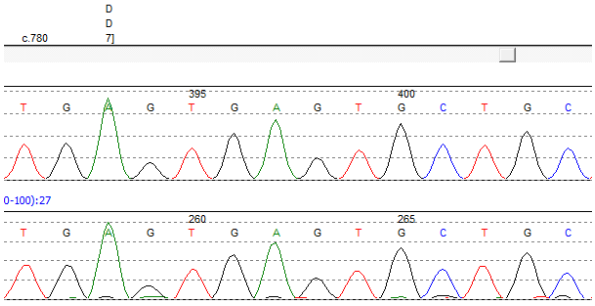
A



B



C



D

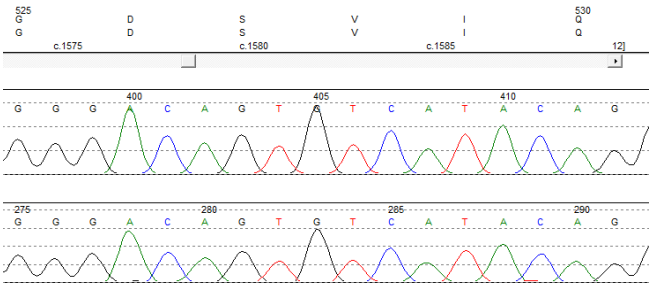


Figure 3.2. Samples of sequencing showing no mutations were found in the genes known to cause hearing loss in Newfoundland, A. *WFS1* Exon 8, B. *TMRPSS3* Exon 4, C. *TMPRSS3* Exon 8, D *PCDH15* Exon 13

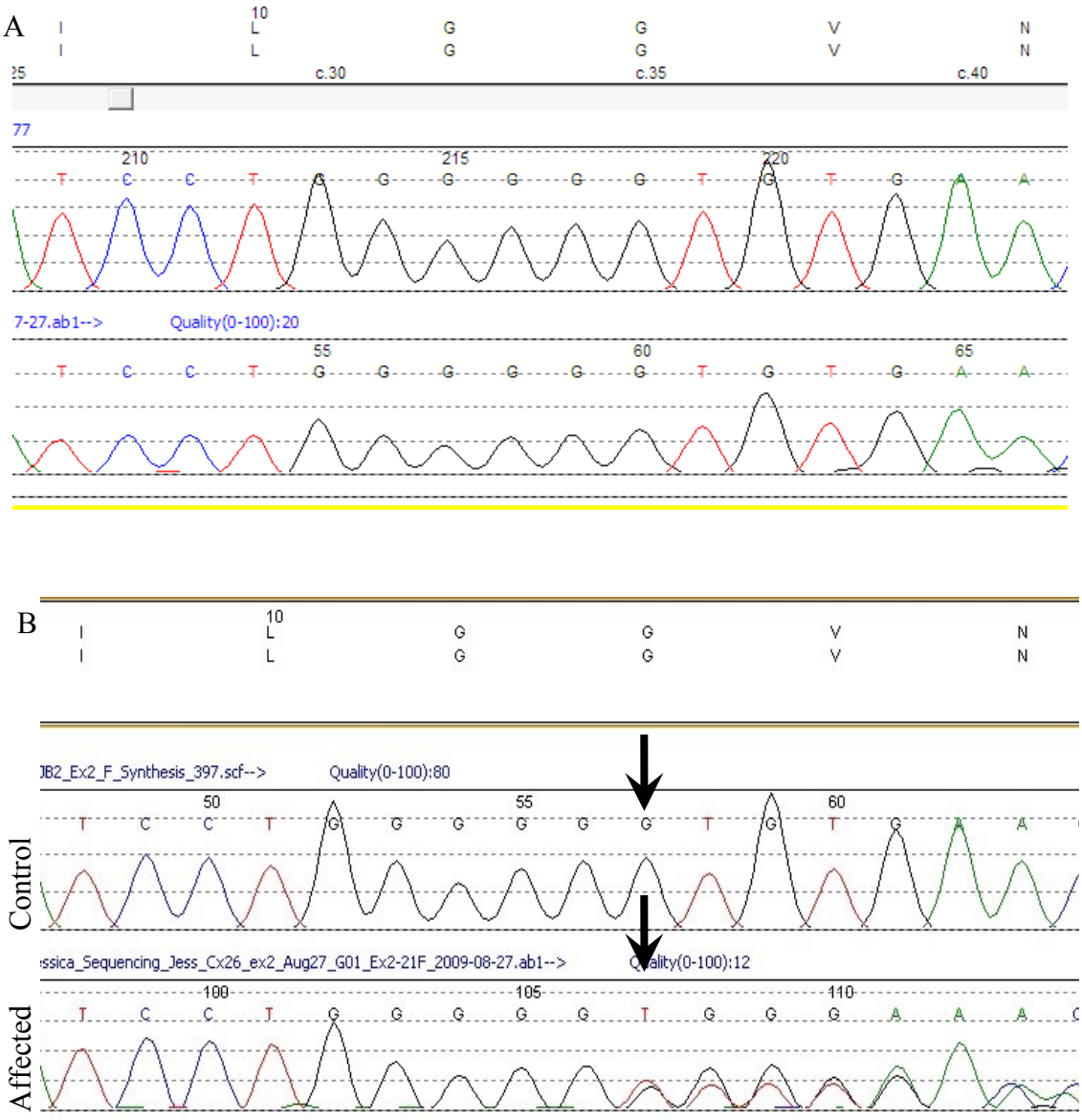
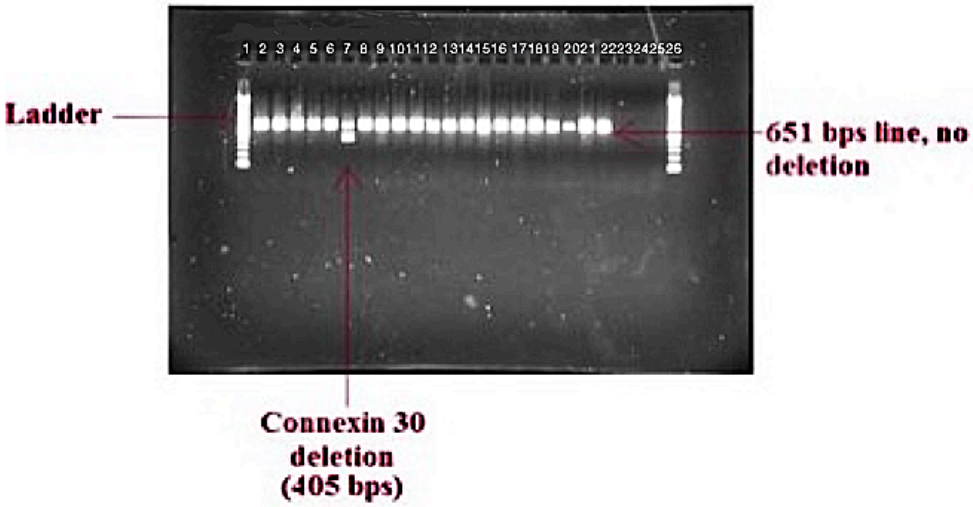


Figure 3.3. An electropherogram showing an individual with a wild type Connexin 26 gene (A) and an electropherogram showing a heterozygous deletion of a guanine (35delG) (arrow) in Connexin 26 in the proband from Family 2155 (B).



Key:

Well 1 – Ladder	Well 10 – FC08-213	Well 19 – JK08 - 229
Well 2 - MT08-239	Well 11 – KS08-195	Well 20 – GH08-233
Well 3 – MR08-325	Well 12- RH08-210	Well 21 – LP09-31
Well 4 – EF08-238	Well 13-08MG1184	Well 22 – EW09-43
Well 5 - MA08-234	Well 14 – JO08-216	Well 23 - Blank
Well 6 – DB08-193	Well 15 – 03MG365B	Well 24 - Blank
Well 7 – RP08-193	Well 16 – BM09-25	Well 25 - Blank
Well 8 - CR09-48	Well 17 – AC08-223	Well 26 - Ladder
Well 9 – DA08-201	Well 18 – JH08-220	

Figure 3.4. An agarose Gel showing heterozygous Connexin 30 deletion in proband from family 2155

For the corresponding family numbers for each DNA sample number see Appendix 5.

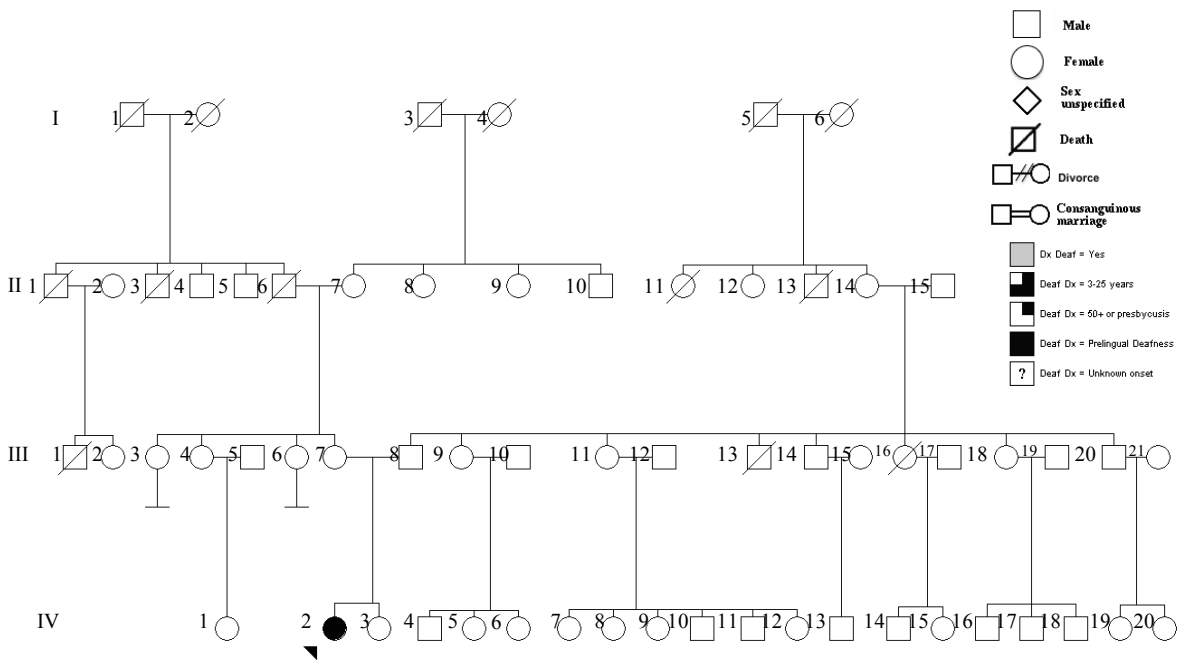


Figure 3.5. The partial pedigree for family 2155 showing autosomal recessive transmission of hearing loss in which the proband (arrow) that carries the connexin 26 35delG mutation as well as the connexin 30 deletion. For the full pedigree see Appendix 4, Figure 1.

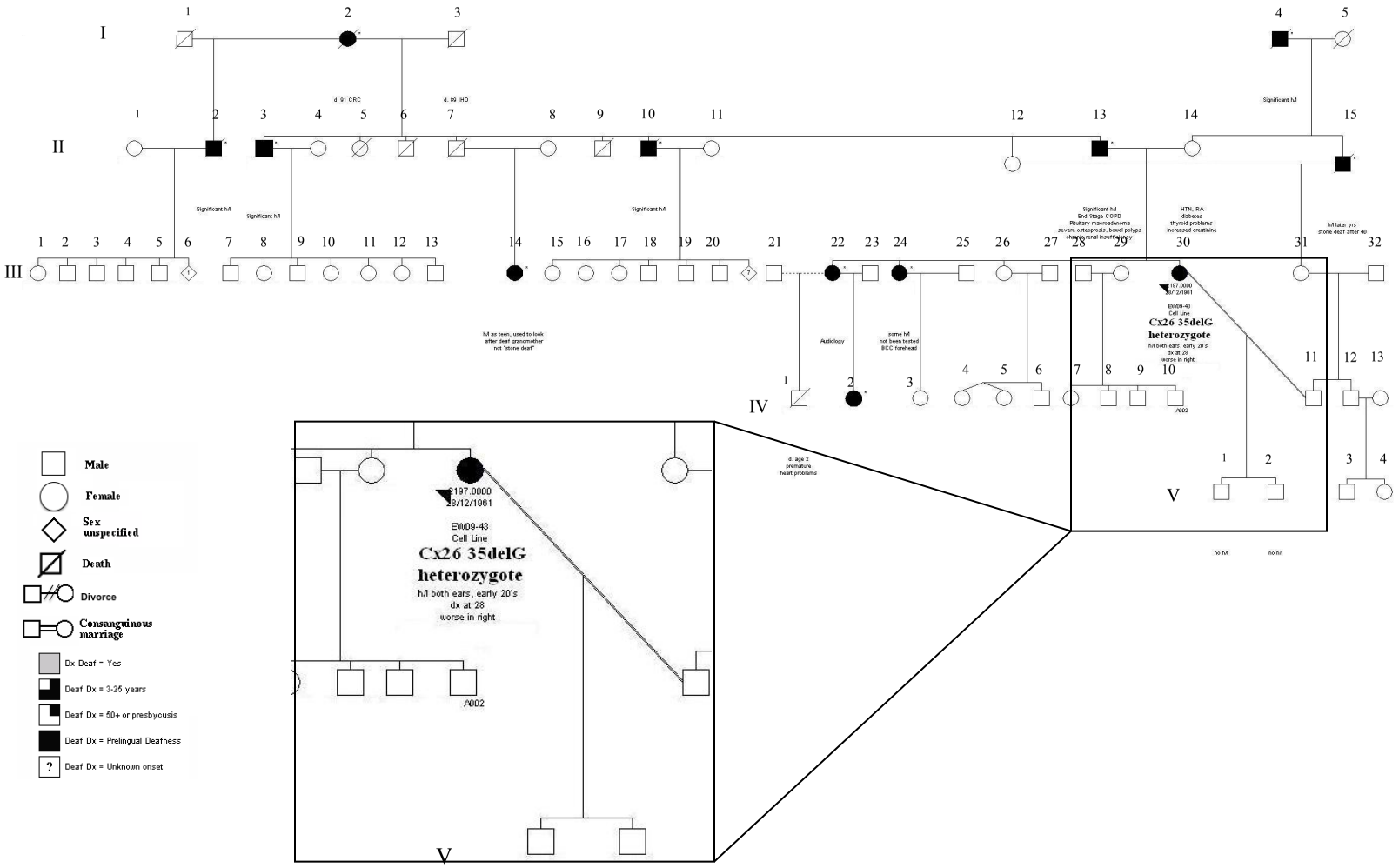


Figure 3.6. The pedigree for family 2197 in which the proband (arrow) carries the connexin 26 35delG.

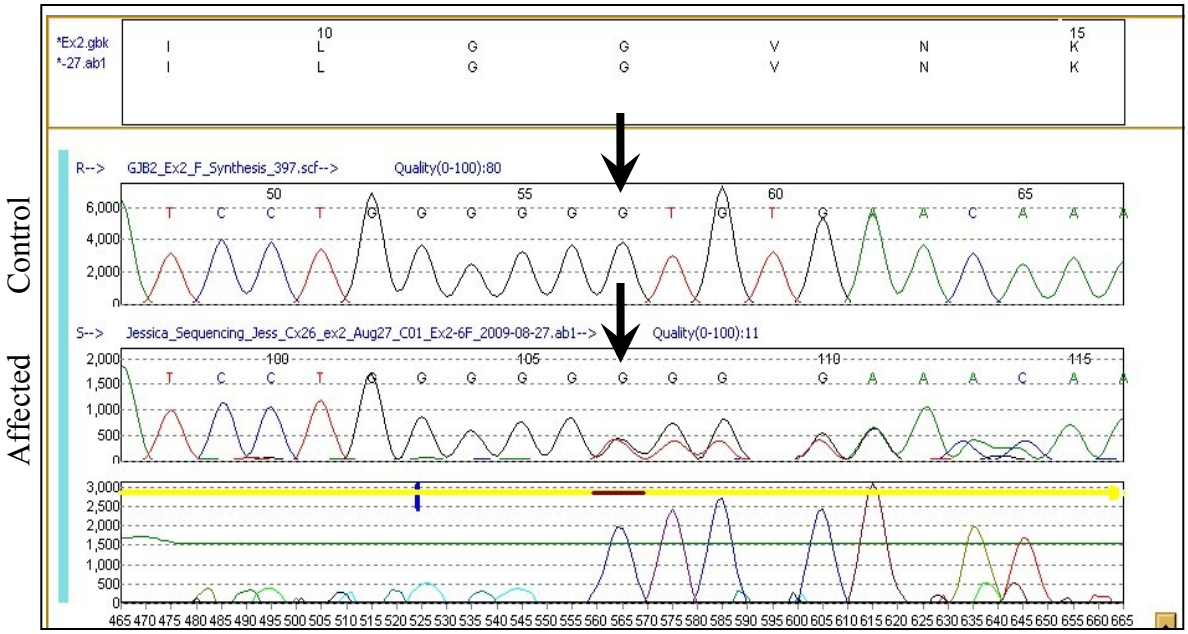


Figure 3.7 An electropherogram showing heterozygous deletion of a guanine (35delG) (arrow) in Connexin 26 in the proband from Family 2197

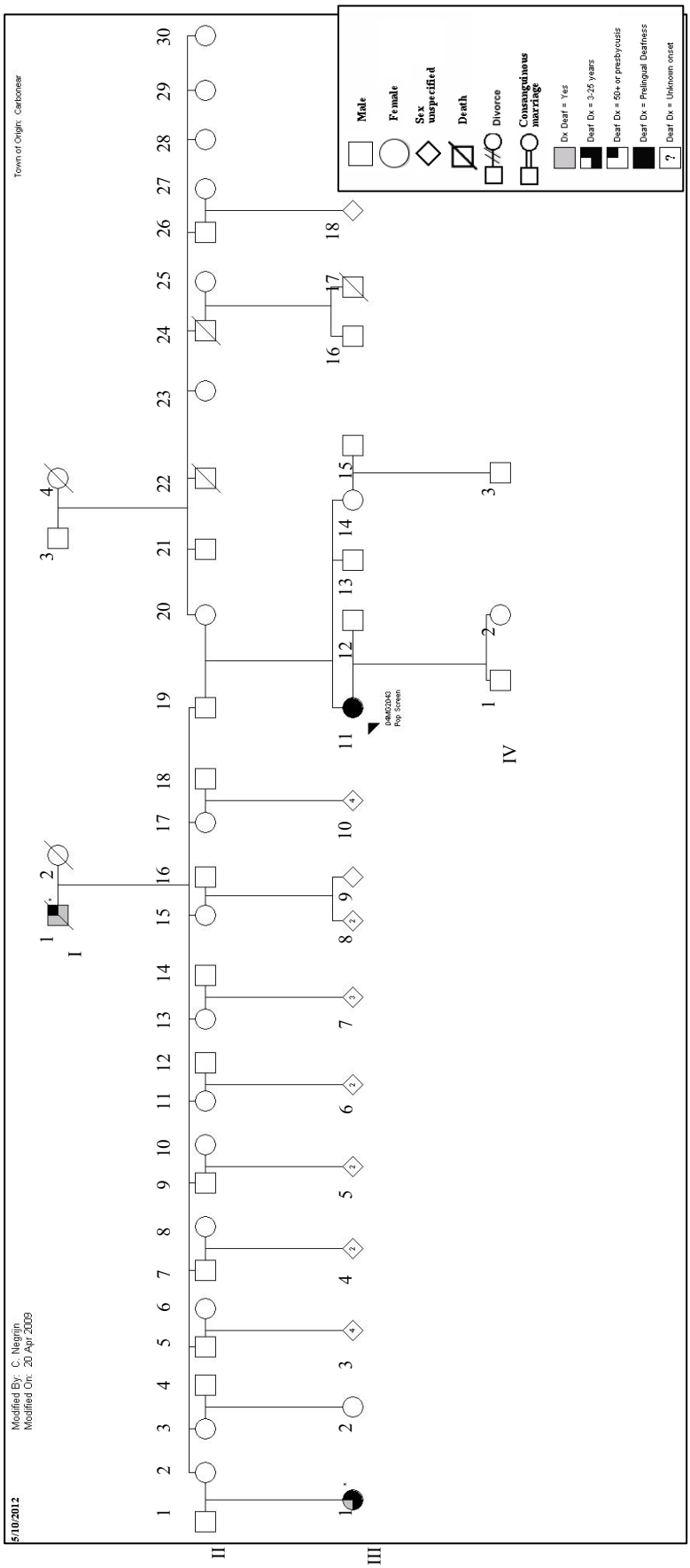


Figure 3.8. The pedigree for the proband from family 2091 in which the F83L variant was detected in connexin 26 exon 2.

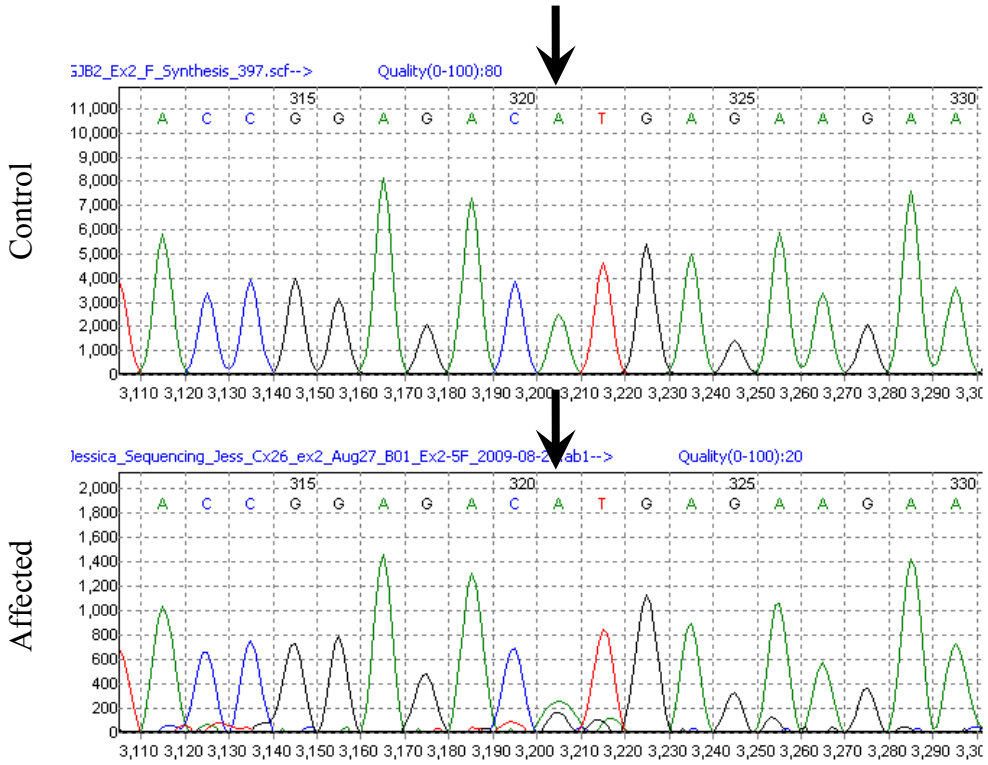


Figure 3.9. An electropherogram showing the nucleotide change c.250A>AG (arrow) that led to the p.F83L variant in Cx26, exon 2, in the proband from family 2091.

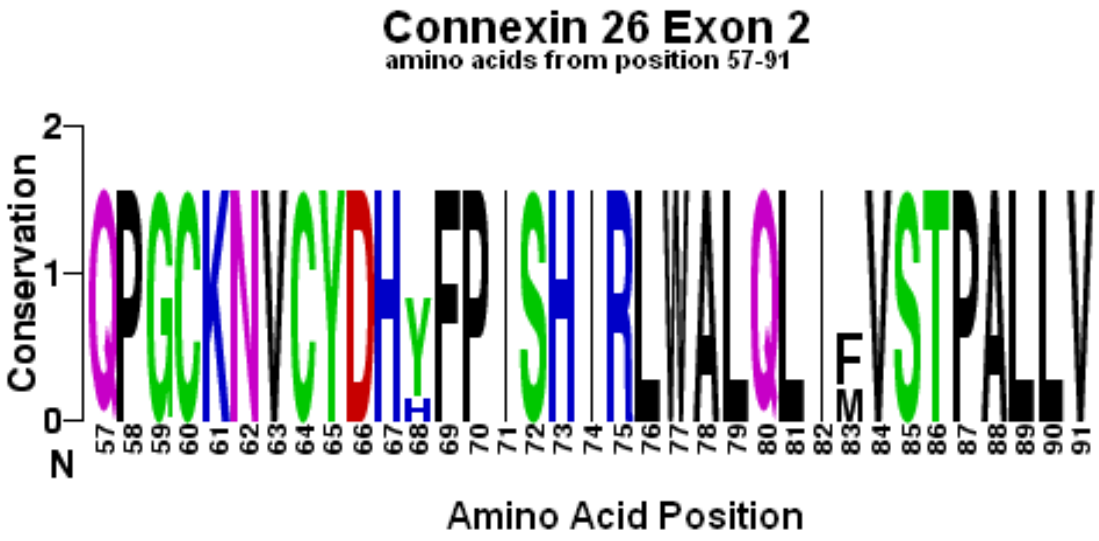


Figure 3.10. A weblogo image pointing out the conservation of the amino acid at position 83. The species used were: Human, Mouse, Norway Rat, Sheep, and Rhesus Monkey.

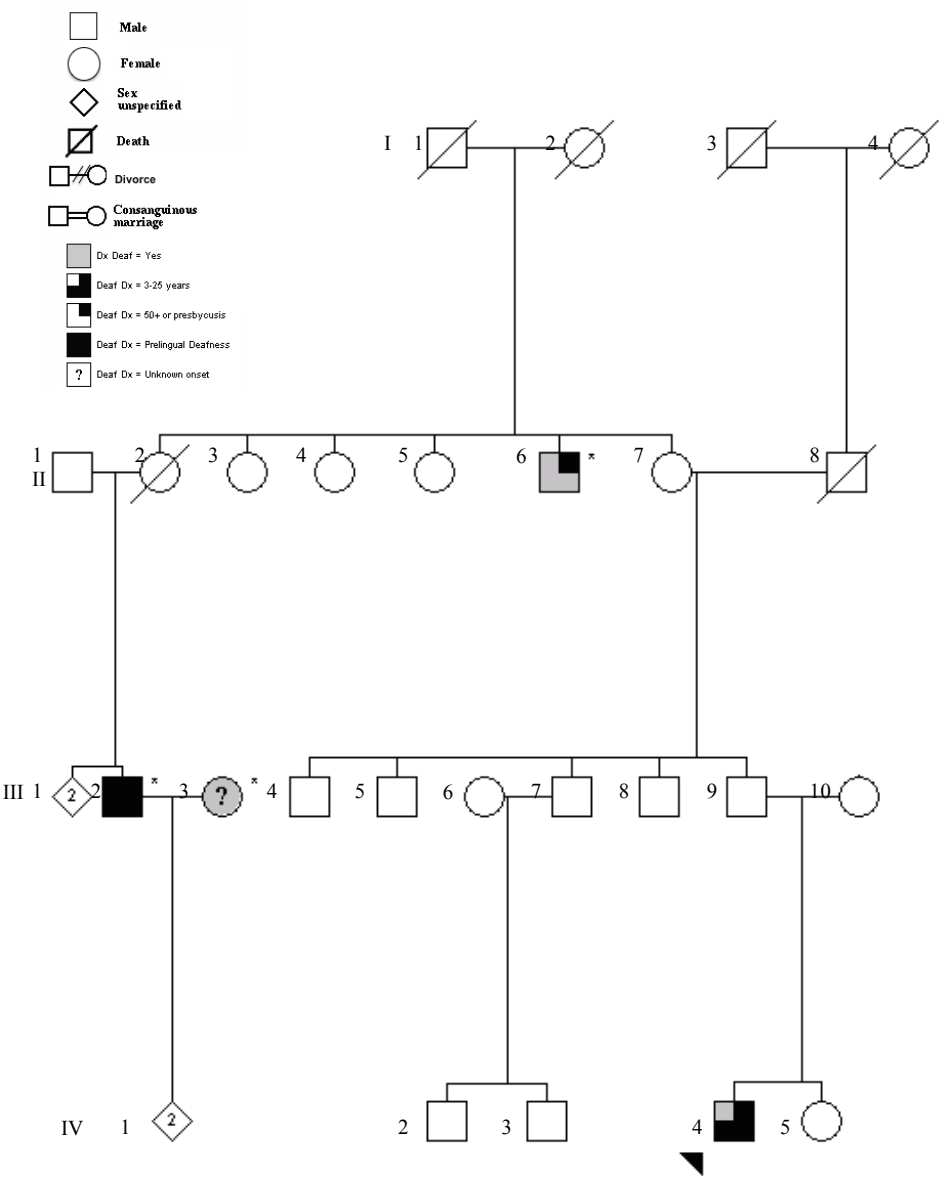


Figure 3.11. A partial pedigree for family 2167 in which the proband (arrow) that carries the G951A mutation in *MTRNR1*. For the full pedigree see Appendix 4, Figure 2.

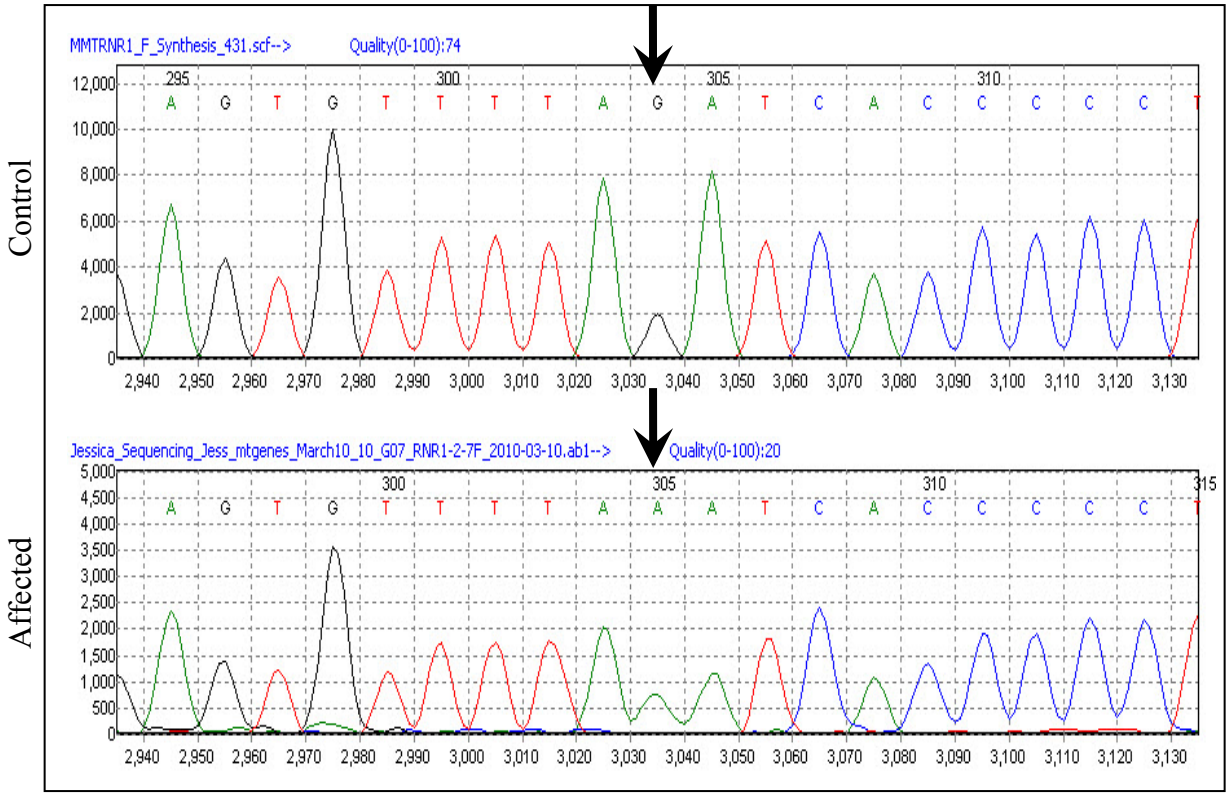


Figure 3.12. An electropherogram showing the homoplasmic change of guanine to adenosine (G951A) in proband from Family 2167

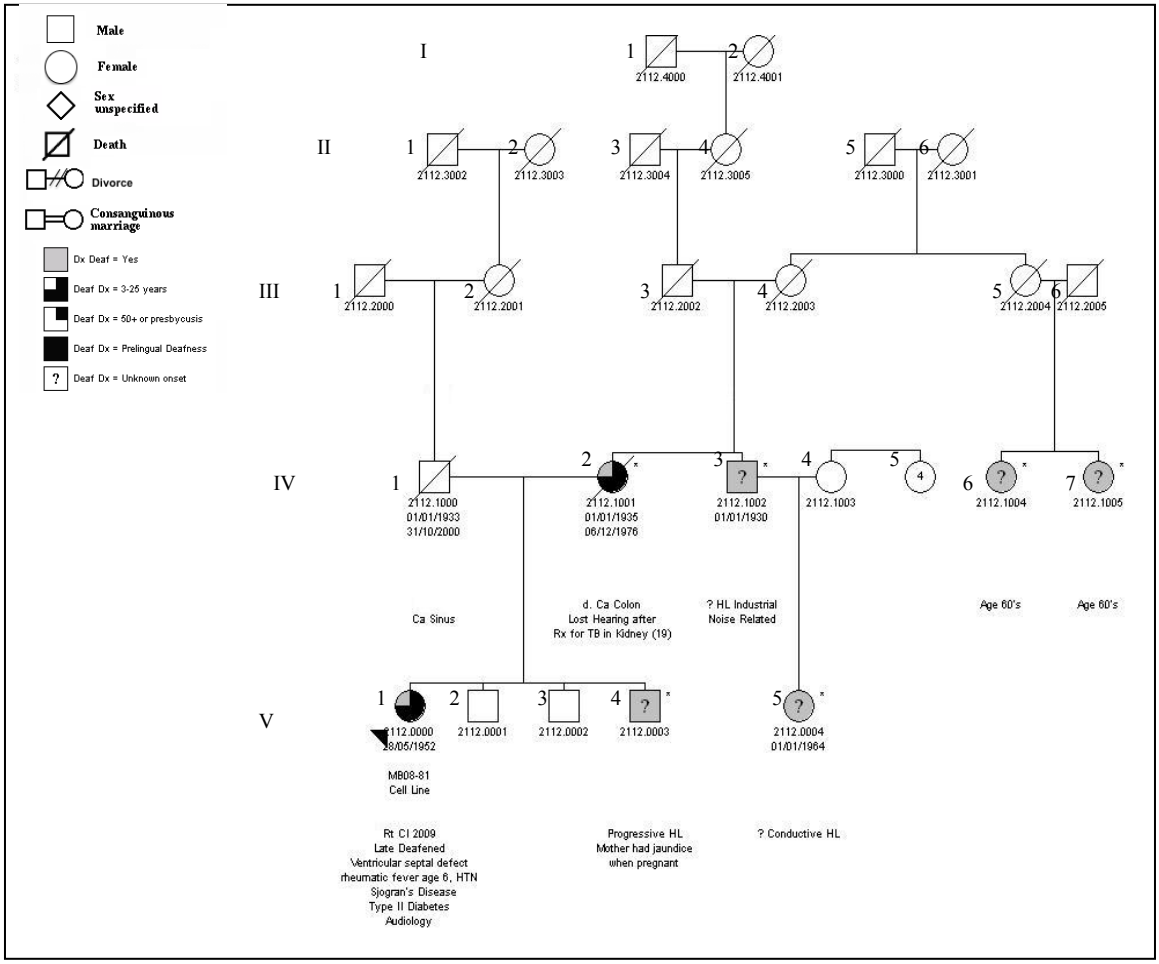


Figure 3.13. The pedigree for family 2112 in which the proband (arrow) carries a homoplasmic A1555G mutation in *MTRNR1*.

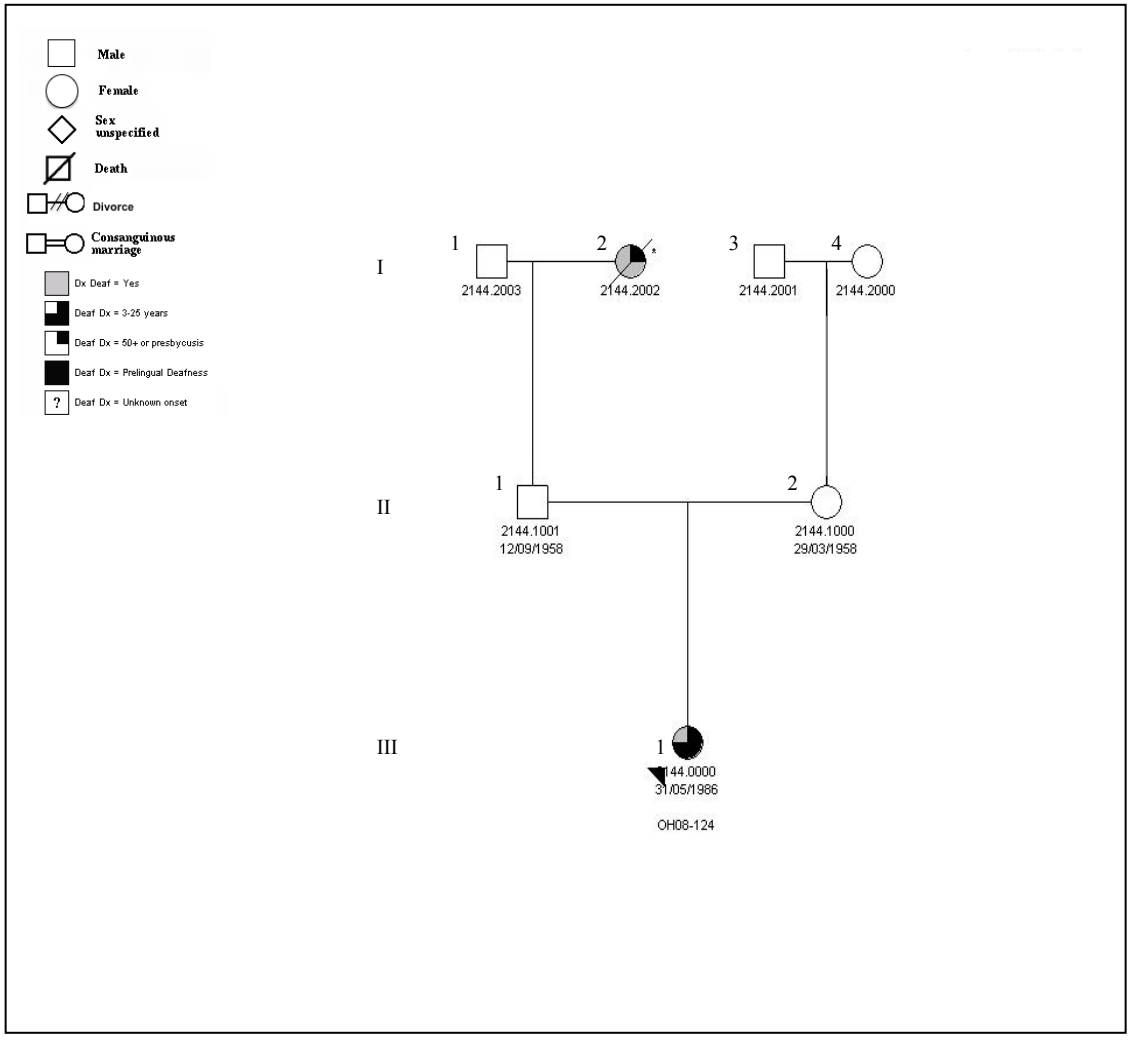


Figure 3.14. The pedigree for family 2144 in which the probands (arrow) carries homoplasmic A1555G mutation in *MTRNR1*.

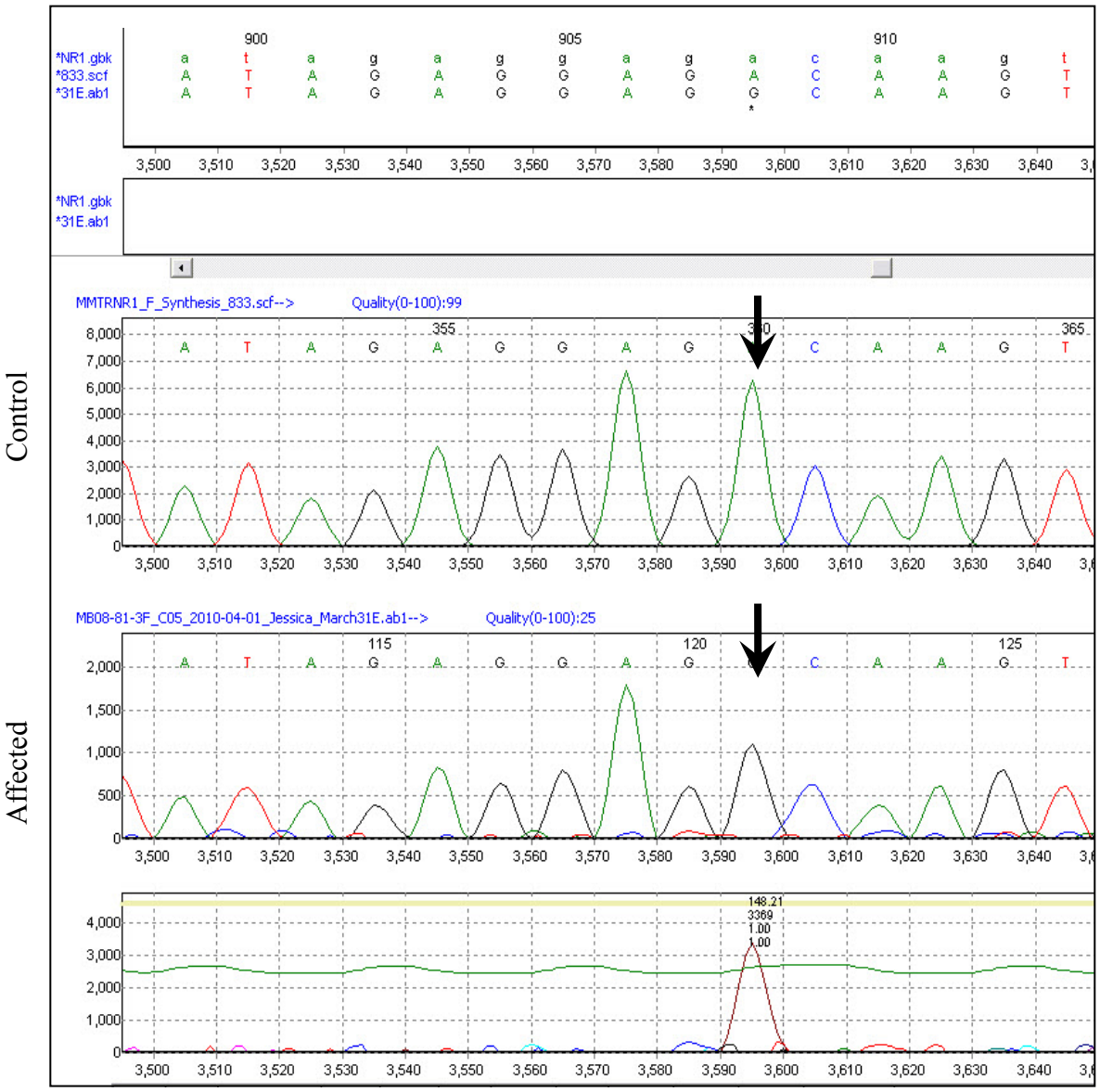


Figure 3.15. An electropherogram showing the homoplasmic change of adenosine to guanine (A1555G) (arrow) in the proband from Family 2112.

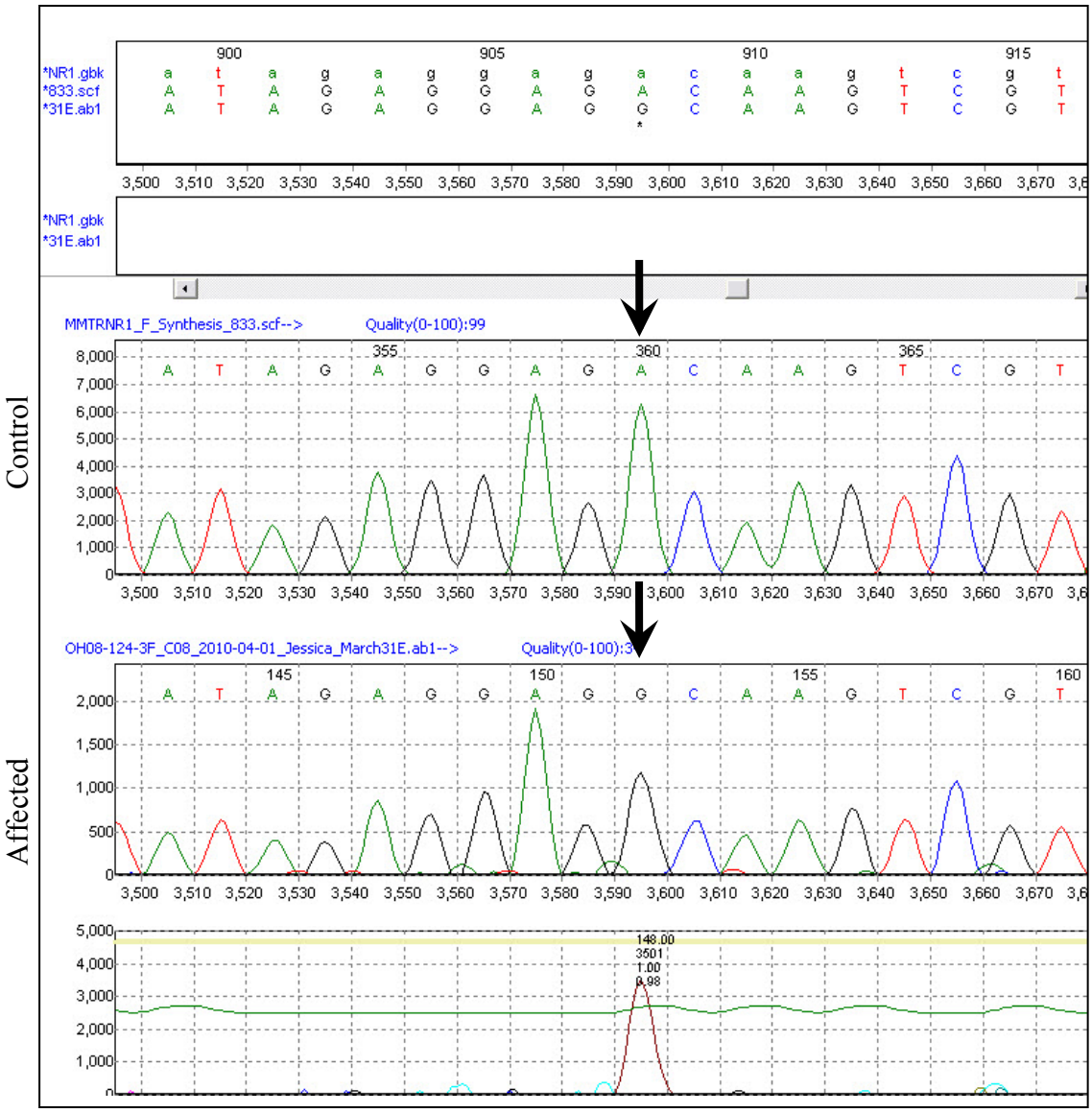


Figure 3.16. An electropherogram showing the homoplasmic change of adenosine to guanine (A1555G) (arrow) in the proband from Family 2144

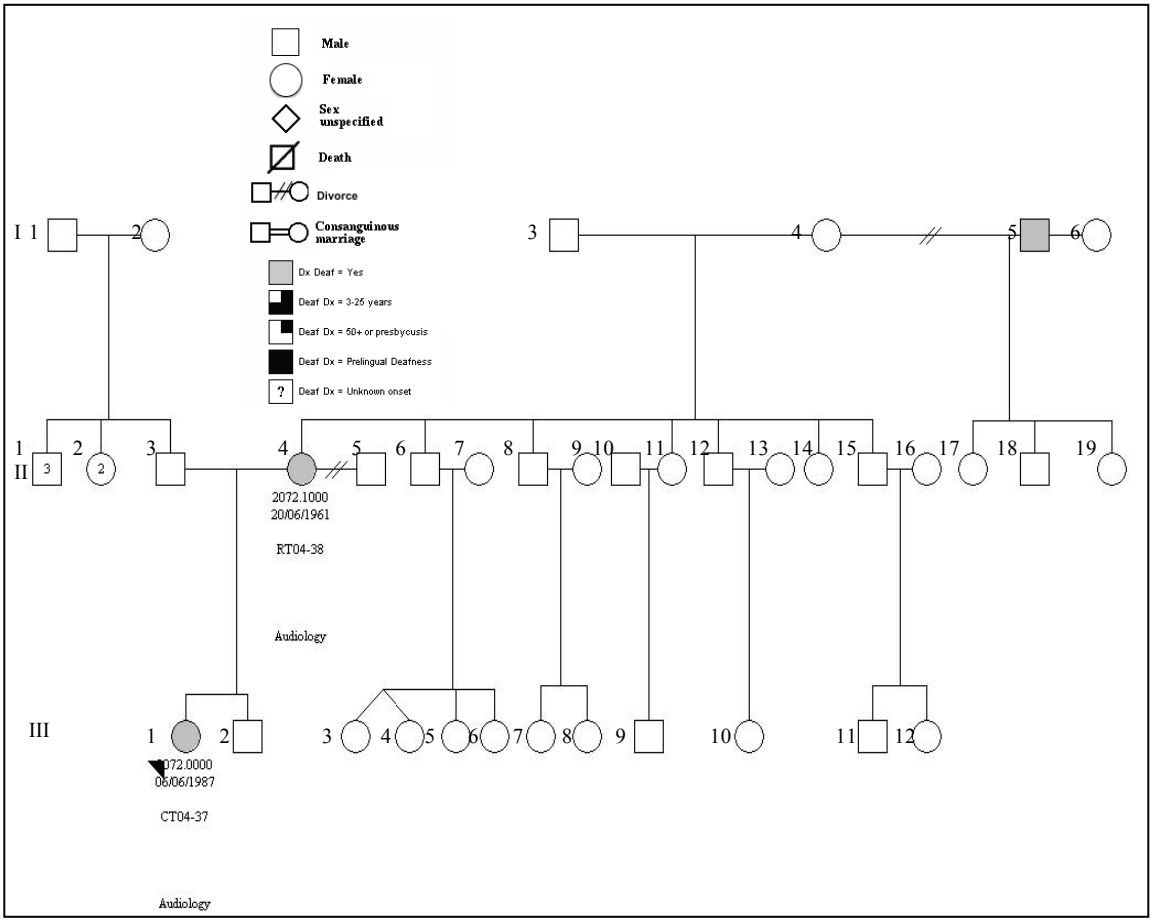


Figure 3.17. The pedigree for family 2072 in which the proband (arrow) and her mother have homoplasmic changes (C150T and T152C) in the control region of mitochondrial genome that caused the mispriming during *MTRNR1* sequencing.

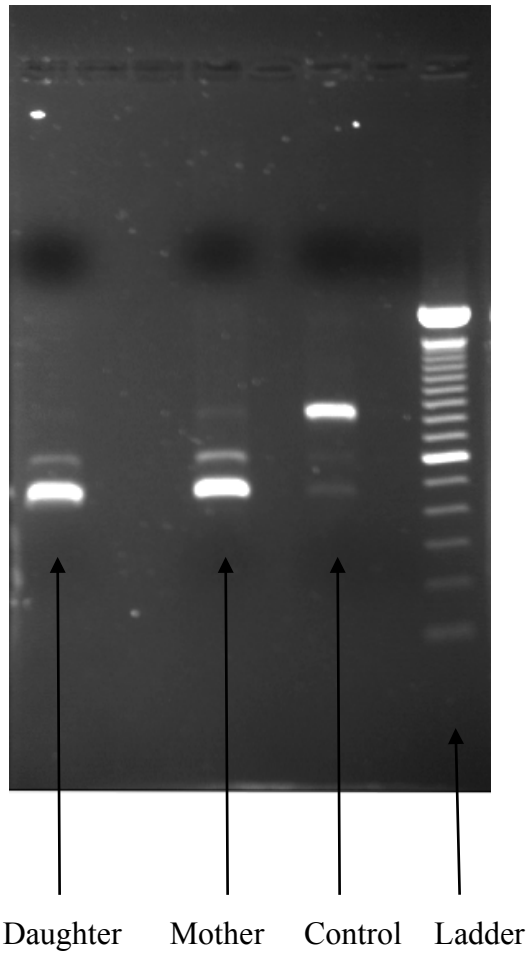


Figure 3.18. An agarose gel showing the apparent deletion in the proband and the proband's mother from family 2072.

Figure 3.19. The wild type sequence of the human mitochondrial genome showing the sequence where the 3' end of the forward primer was initially supposed to attach (pink) and the similar sequence 349 bps downstream from that point (light blue). Because it is important for primers to be specific, the SNPs in the proband's mitochondrial genome at the binding site caused the primer to bind 349 bps away from the intentional site, therefore creating an amplicon that was approximately 349bp shorter.

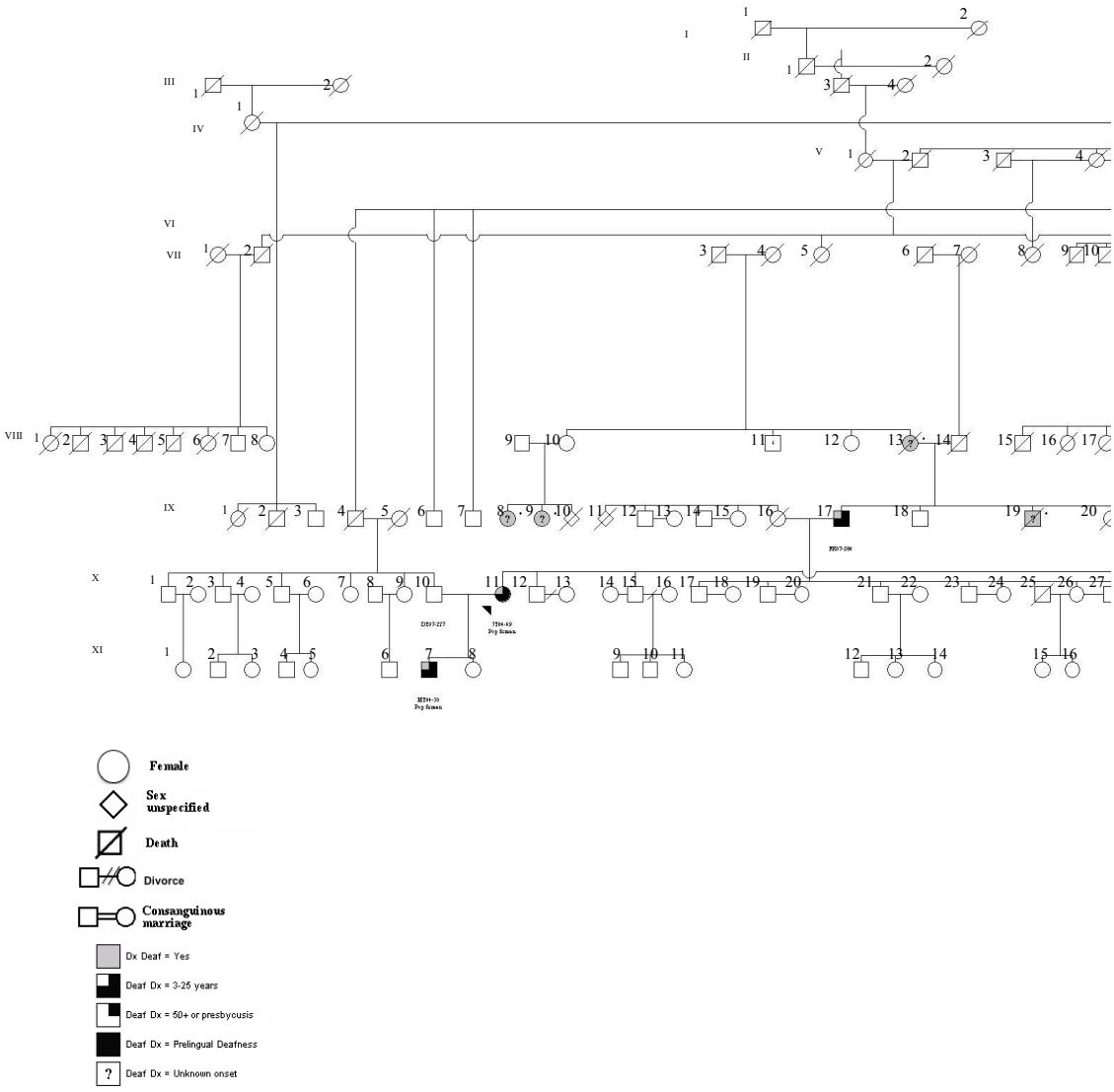
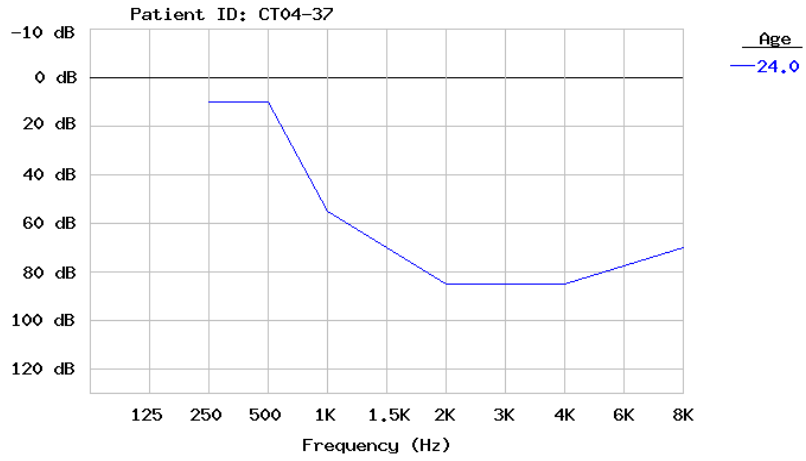
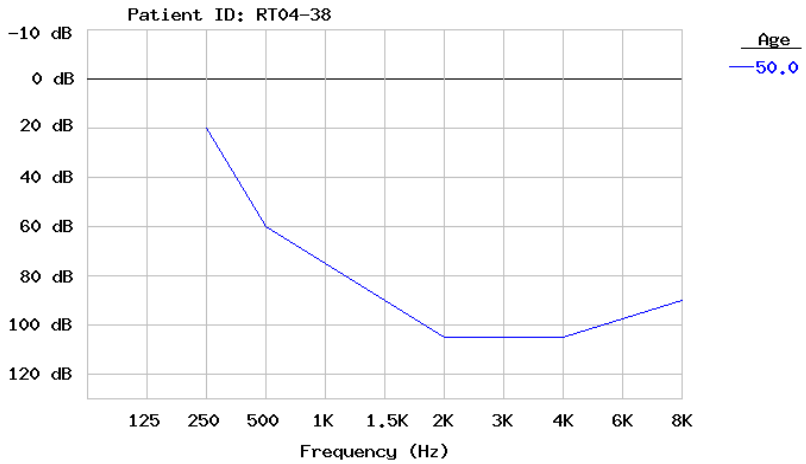


Figure 3.20. A partial pedigree for family 2010 in which the proband (black arrow) and 4 of her family members have homoplasmic changes (C150T and T152C) in the control region of mitochondrial genome that caused the mispriming during *MTRNR1* sequencing. The proband from also has the R141W mutation in *TMC1*.

A



B



C

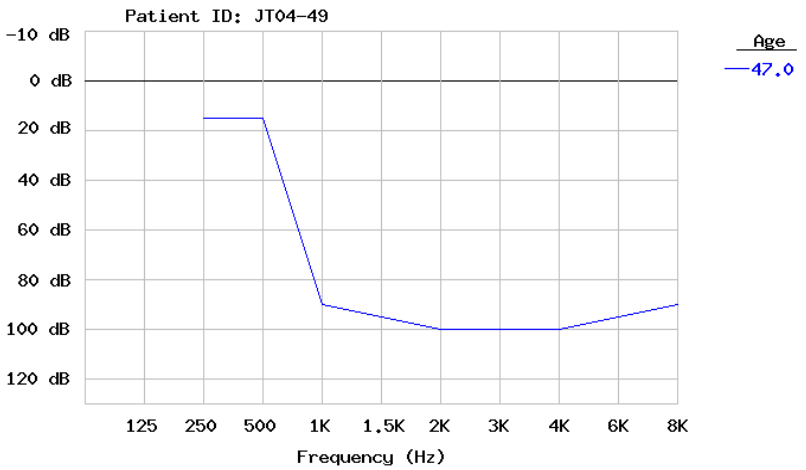


Figure 3.21. Audiograms for the proband (A) and her mother (B) from family 2072 and the audiogram for the proband (C) from family 2010. All showing high frequency hearing loss.

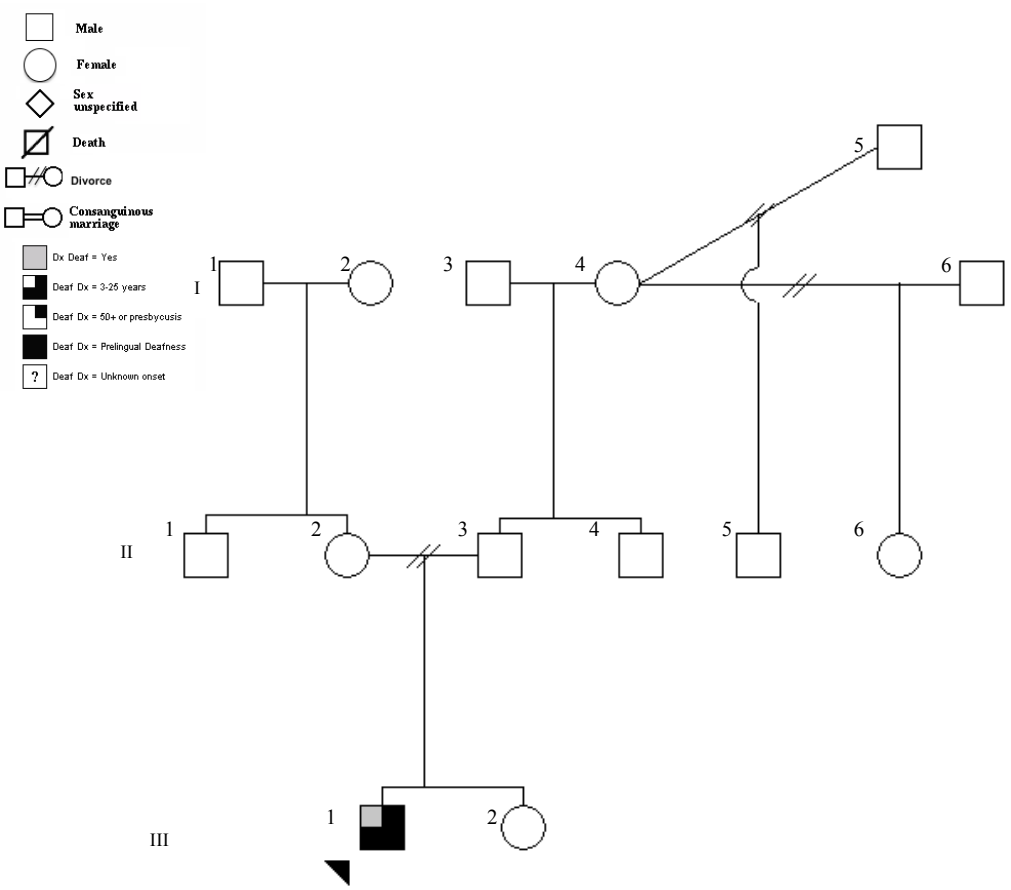


Figure 3.22. The pedigree for family 2078. The proband of this family was found to have a heterozygous c.94C>T:p.R32W mutation in connexin 31.

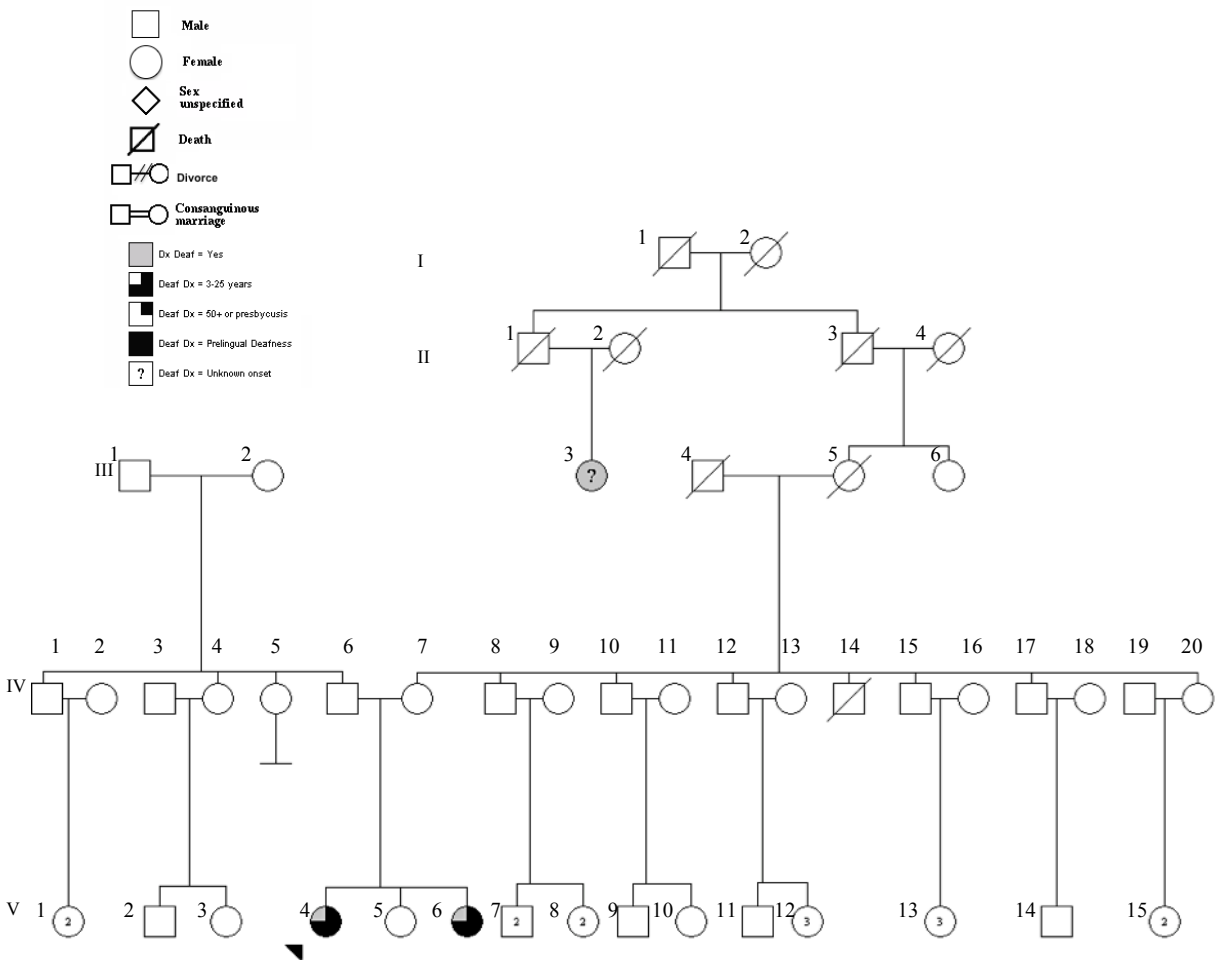


Figure 3.23. The pedigree for family 2075. The proband of this family was found to have a heterozygous c.94C>T:p.R32W mutation in connexin 31.

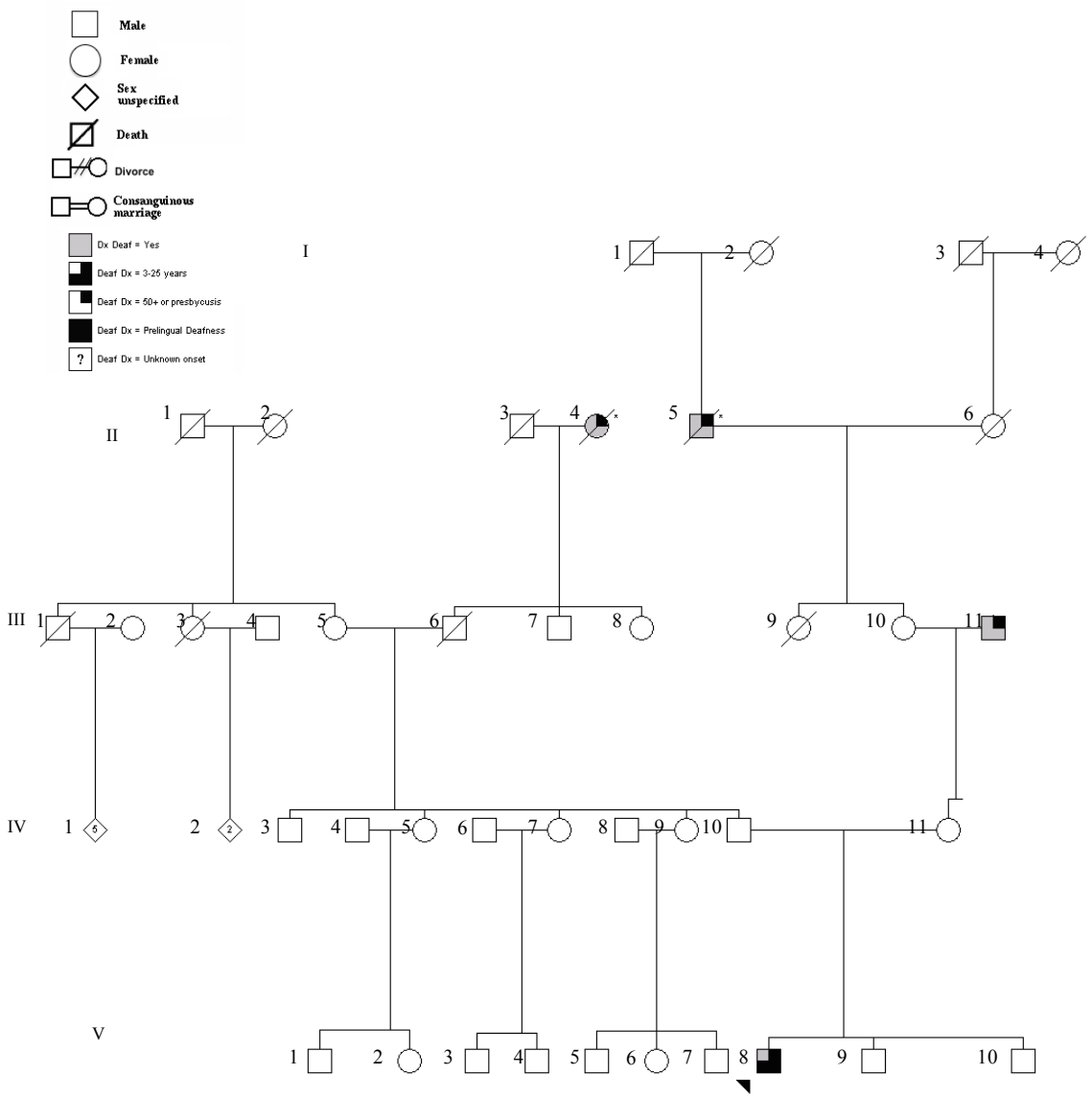


Figure 3.24. A partial pedigree for family 2097. The proband of this family was found to have a heterozygous c.94C>T:p.R32W mutation in Cx31. For the full pedigree see Appendix 4, Figure 3.

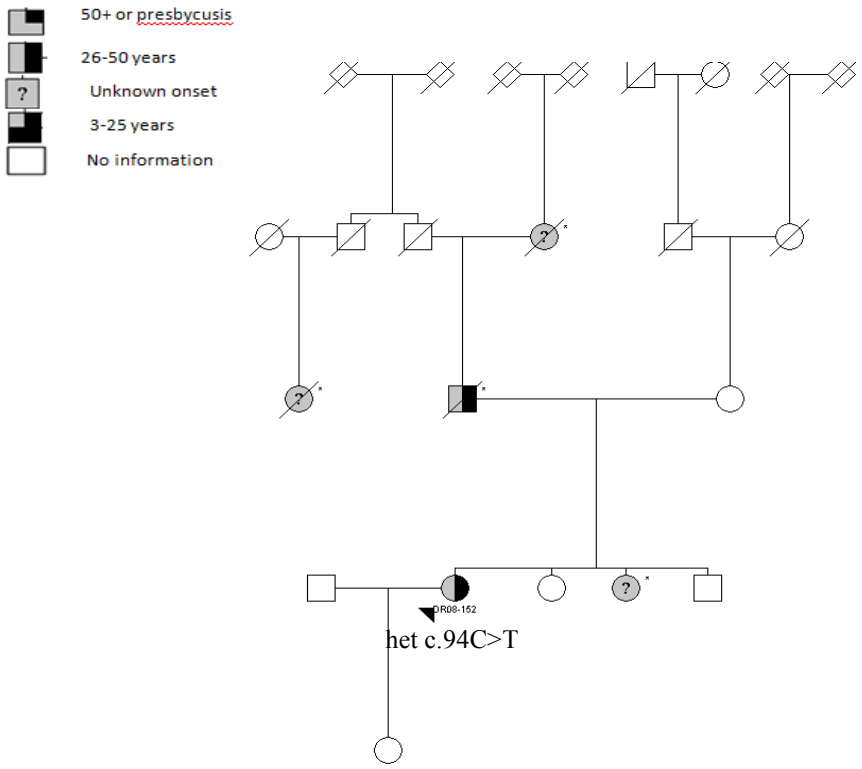


Figure 3.25. The pedigree for family 2156. The proband of this family was found to have a heterozygous c.94C>T:p.R32W mutation in connexin 31.

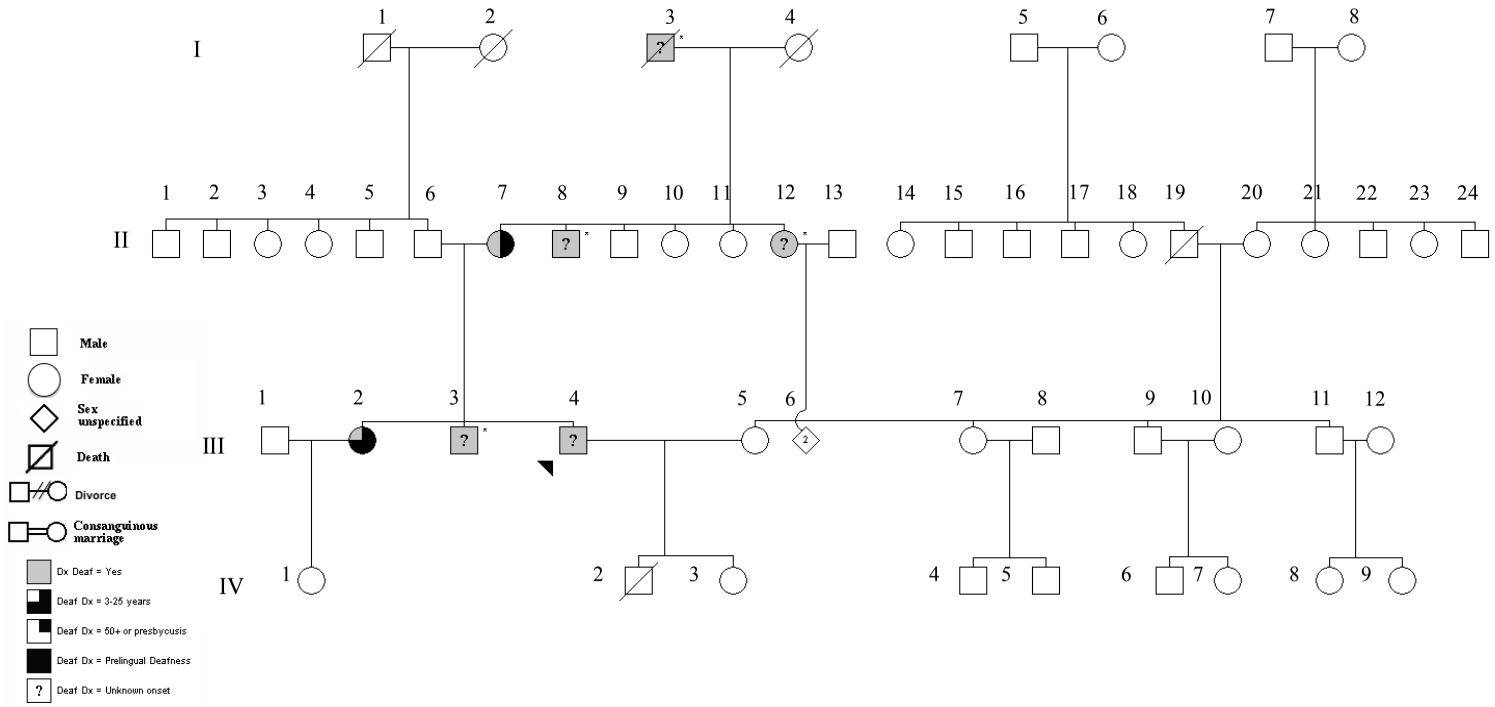


Figure 3.26. A pedigree for family 2083. The proband of this family was found to have a heterozygous V37M mutation in connexin 31.

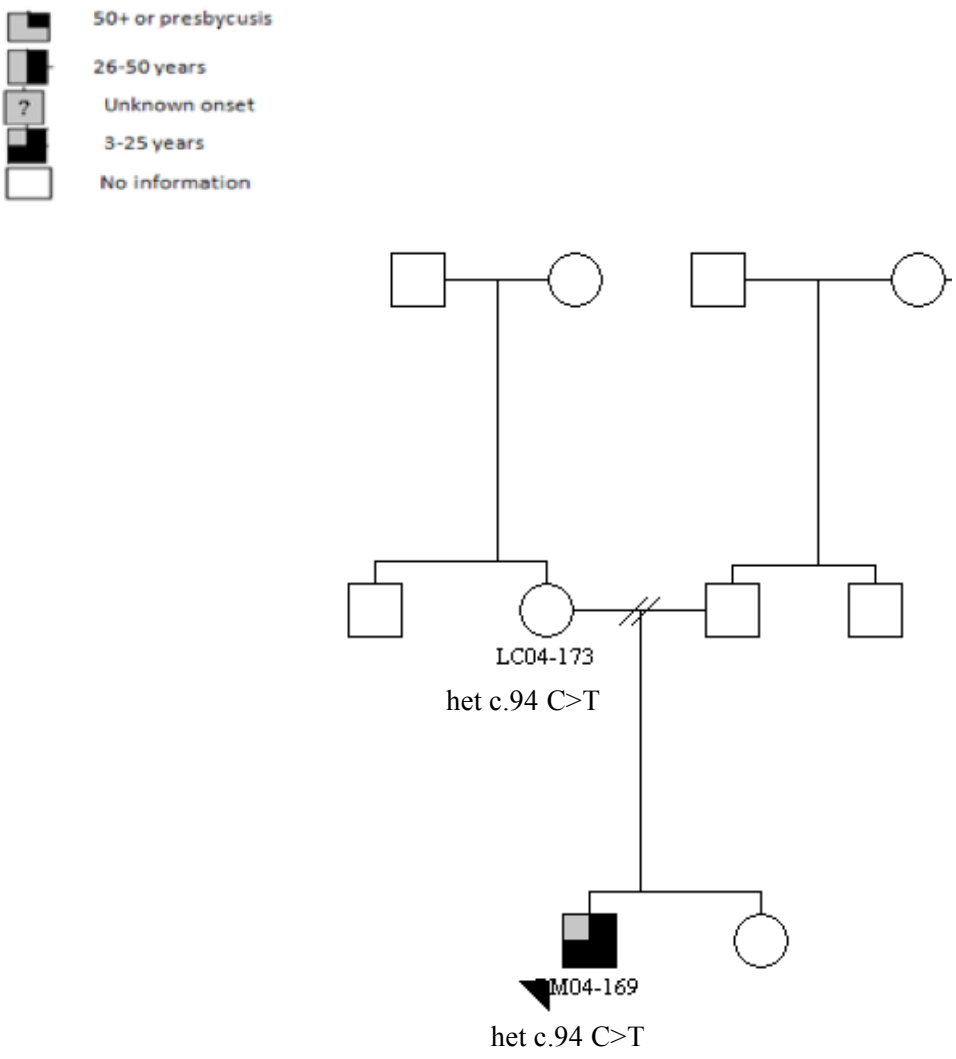


Figure 3.27 The pedigree for family 2078 showing the family members with the heterozygous c.94 C>T mutation.

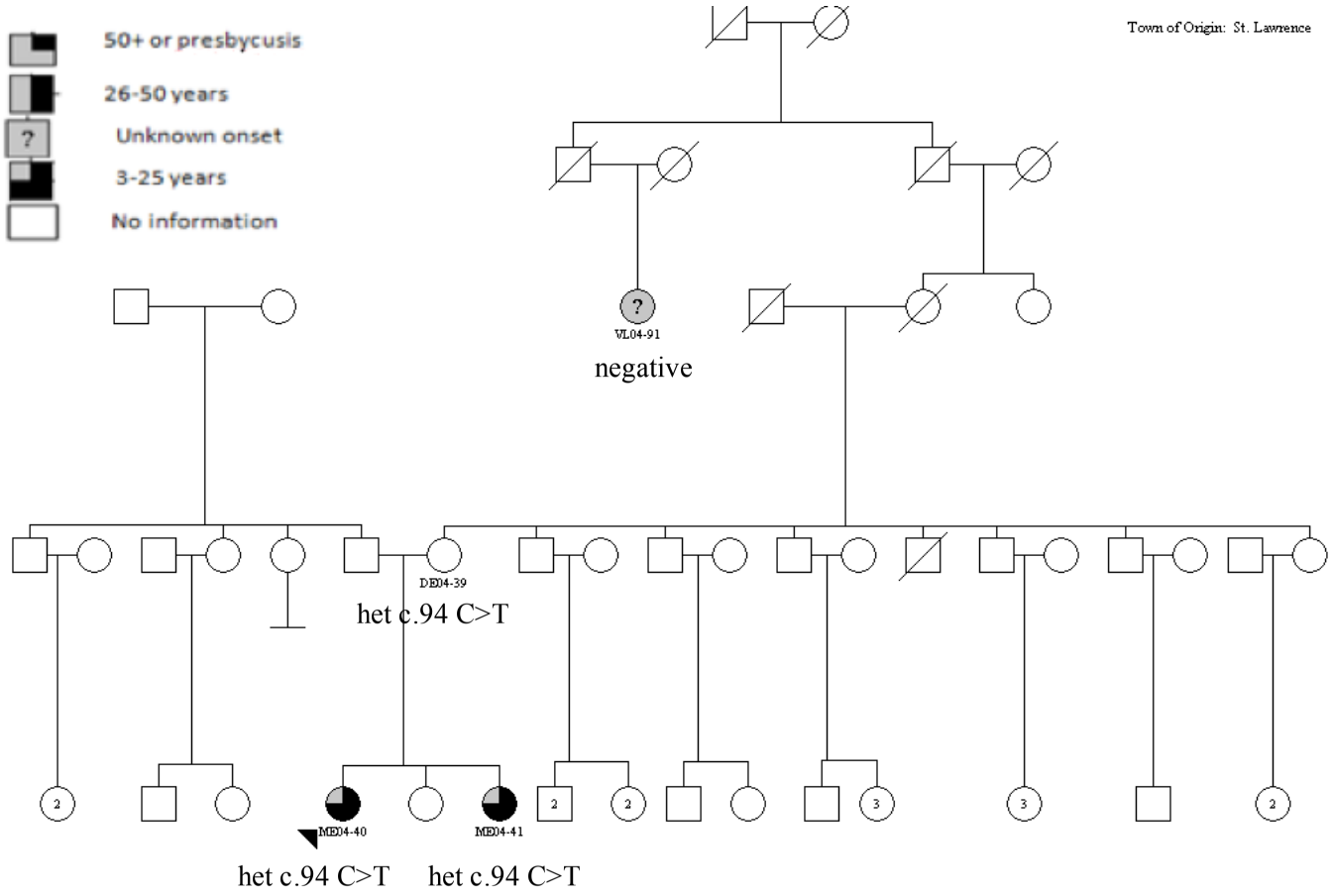


Figure 3.28 The pedigree for family 2075 showing the family members with the heterozygous c.94 C>T mutation.

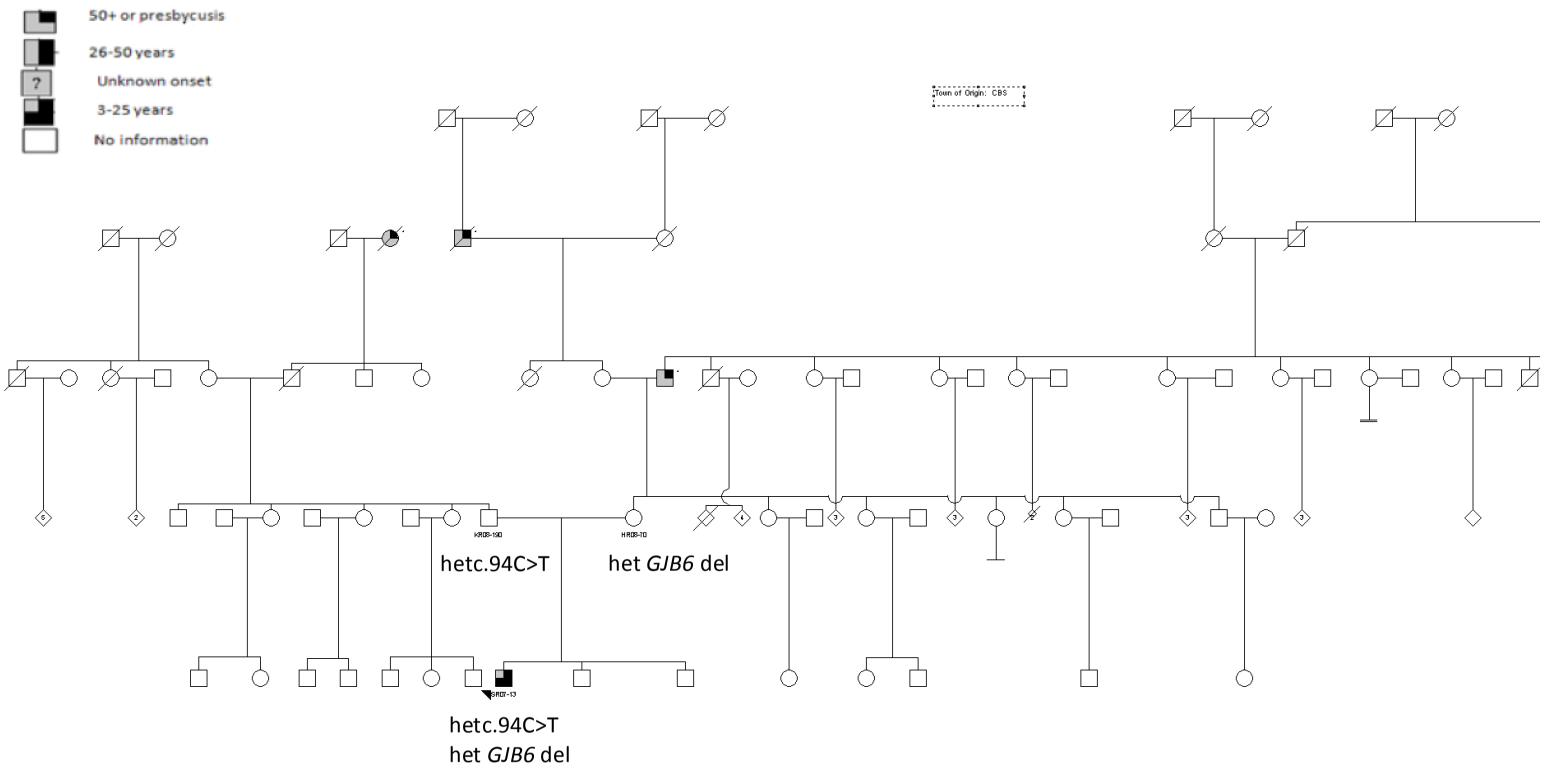


Figure 3.29 The pedigree for family 2097 showing the family members with the heterozygous c.94 C>T mutation.

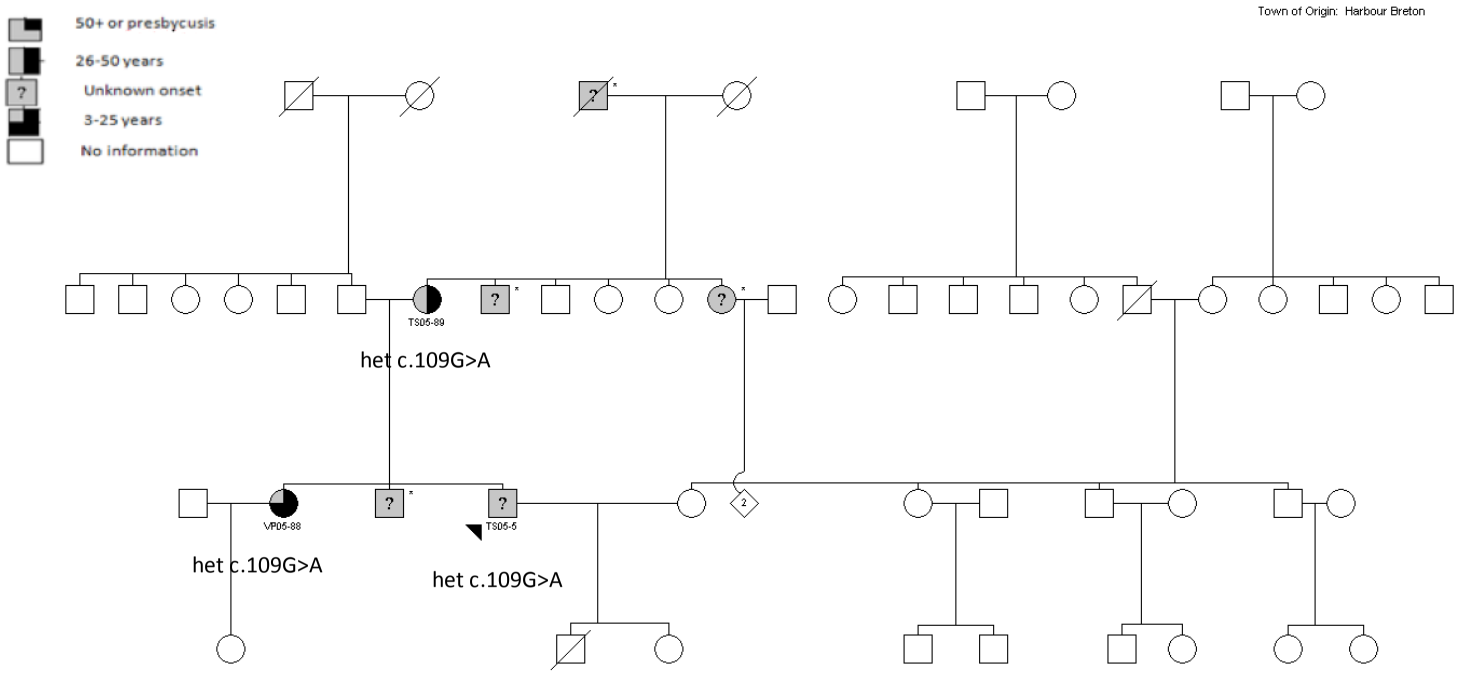


Figure 3.30 The pedigree for family 2083 showing the family members with the heterozygous c.109 G>A mutation.

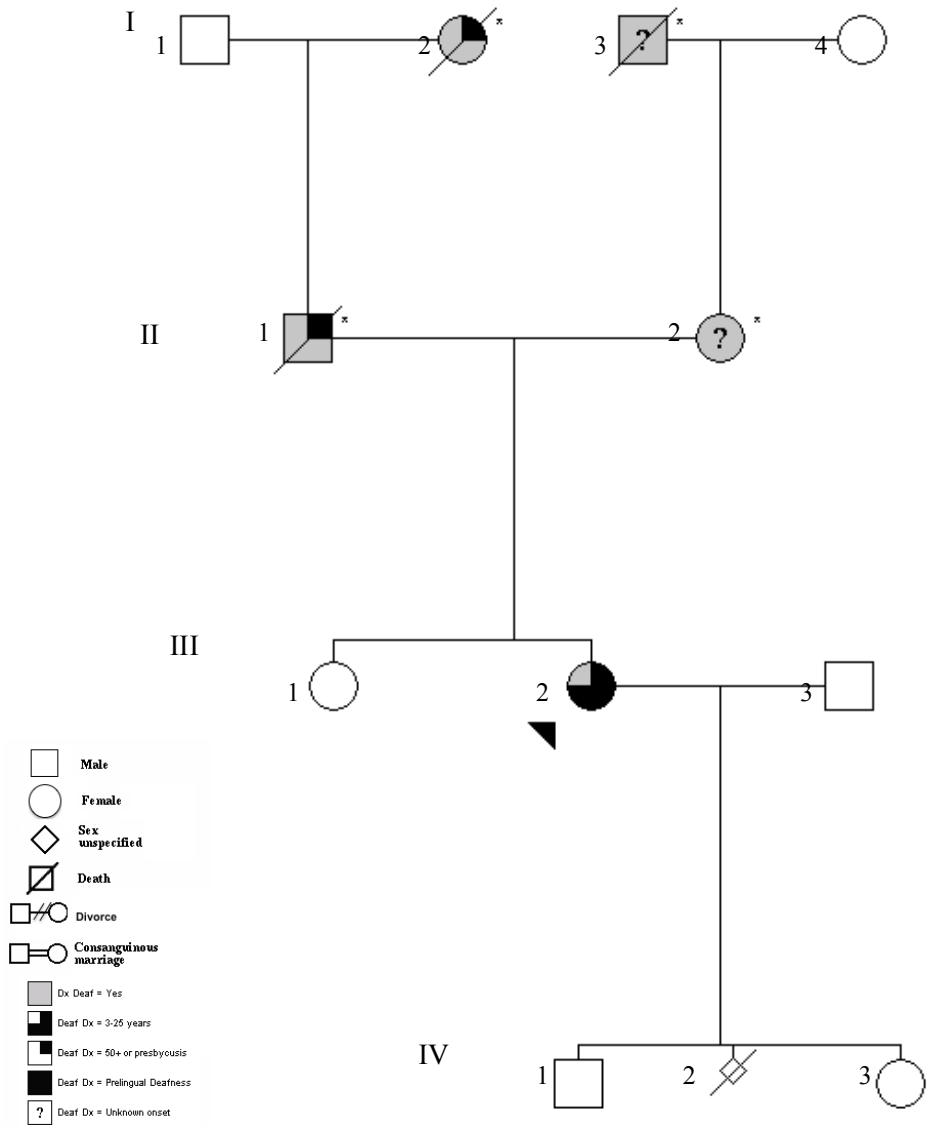


Figure 3.31. The pedigree for family 2124. The proband of this family was found to have a heterozygous G182D mutation in *TMC1*.

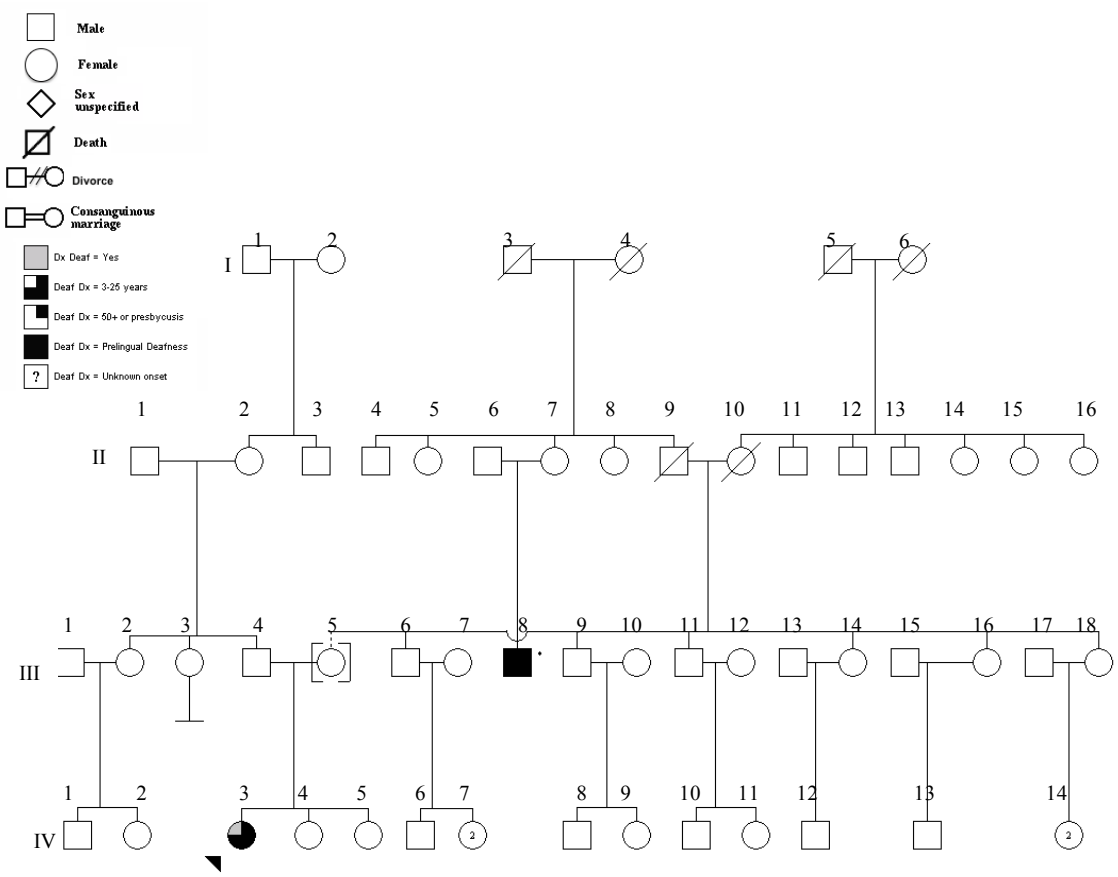


Figure 3.32. The pedigree for family 2065. The proband from this family has the c.1763+A>G mutation in *TMC1*.

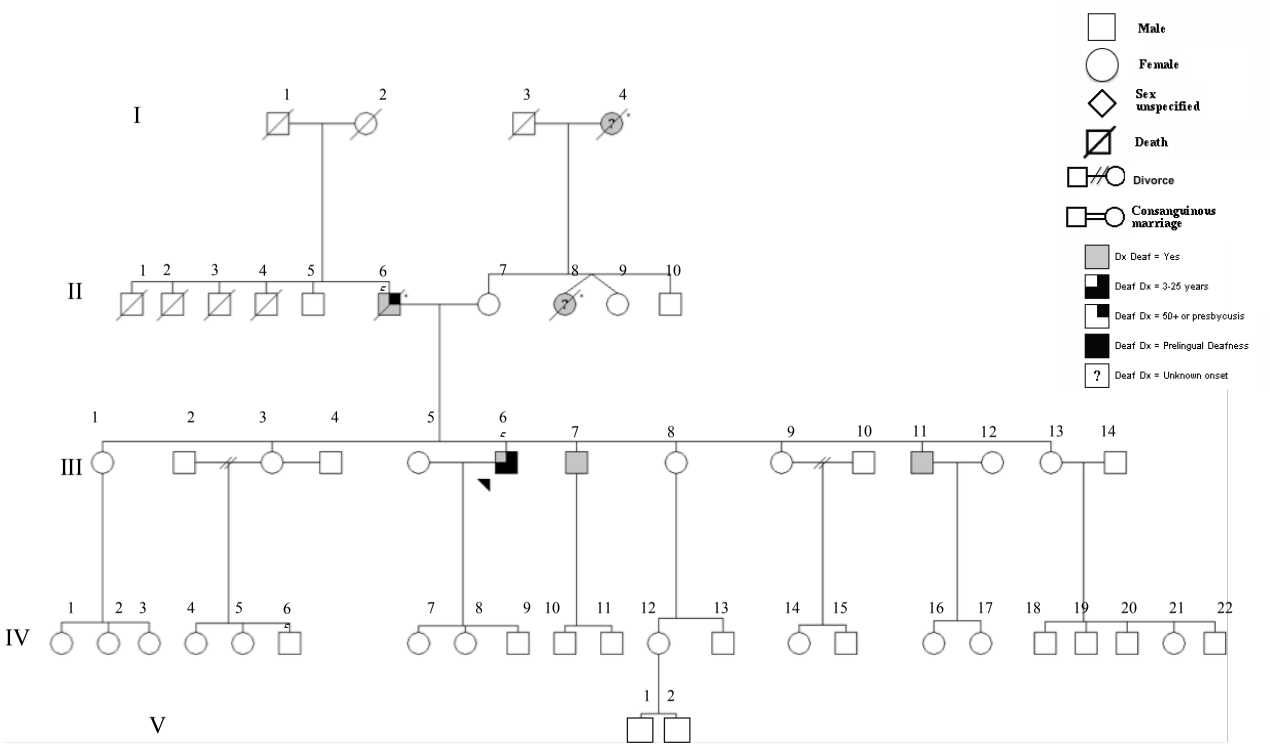


Figure 3.33. The pedigree for family 2146. The proband in this family has the R141W mutation in *TMC1*

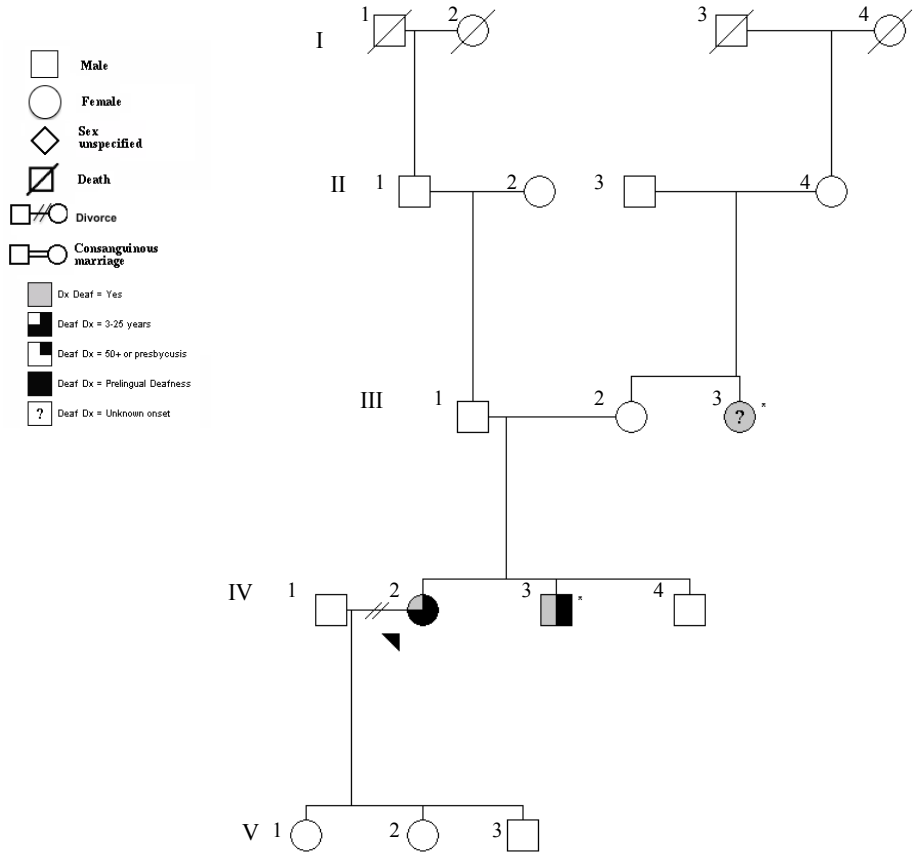


Figure 3.34. The pedigree for family 2177. The proband from this family has the R141W mutation in *TMC1*.

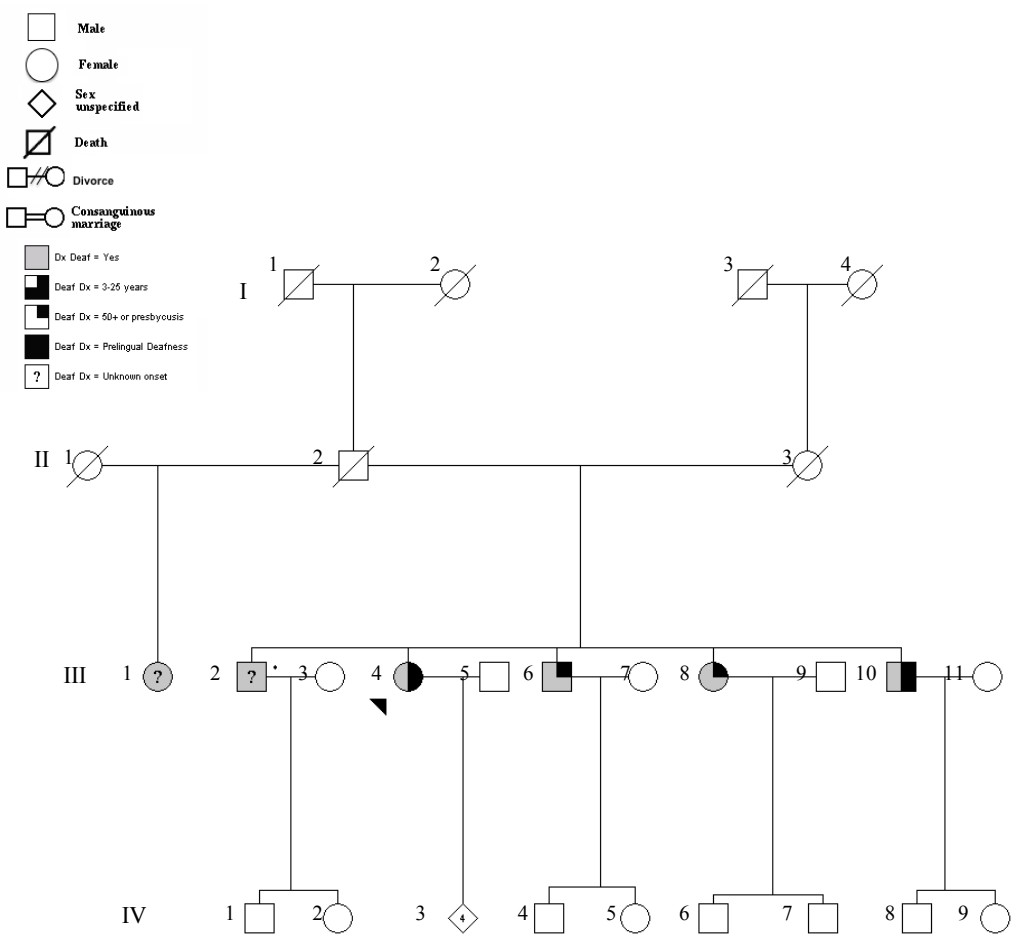


Figure 3.35. The pedigree for family 2092. The proband from this family has the R141W mutation in *TMC1*.

Table 1. Prediction of the Connexin 26 F83L Variant Effect.

Program:	SIFT	Polyphen	SNPs3D	PMut
F83L Variant:	Tolerated	Benign	Tolerated	Neutral

Five bioinformatic programs were used to predict the effect of the F83L mutation. The overall evaluation predicts this mutation to be benign.

Table 2. *MTRNR1* polymorphisms found in 20 deaf probands from NL.

***MTRNR1* Polymorphisms**	**Number of Probands**
A750G [1]	20/20
A1438G[1]	18/20
G930A[1]	1/20
A751G[2]	1/20
T1189C[1]	1/20
G951A[1]	1/20

1-Guaran *et al*., 2013; 2 – Li *et al.* 2004

			MITOCHONDRIAL D LOOP REGION aka VARIABLE REGION aka CONTROL REGION												
FAMILY #	Relationship	PROBAND	T72C	A73G	T119C	C140T	T146C	C150T	T152C	A153G	G185A	A188G	A189G	C194T	T195C
2010	proband	JT04-49	T	G	T	C	T	T	C	A	G	A	A	C	T
2010	sister	AS07-279	T	G	T	C	T	T	C	A	G	A	A	C	T
2010	sister	EH07-277	T	G	T	C	T	T	C	A	G	A	A	C	T
2010	mother	EK07-281	T	G	T	C	T	T	C	A	G	A	A	C	T
2010	sister	GC08-20	T	G	T	C	T	T	C	A	G	A	A	C	T
2010	paternal uncle	FK07-281	T	A	T	C	T	C	T	A	G	A	A	C	T
2072	proband	CT04-37	T	G	T	C	T	T	C	A	G	A	A	C	T
2072	mother	RT04-38	T	G	T	C	T	T	C	A	G	A	A	C	T
		DJ08-51	T	G	T	C	T	C	T	A	G	A	A	C	T
		EM08-154	T	G	T	C	T	C	T	A	G	A	A	C	T
		GP09-103	T	G	T	C	T	C	T	A	G	A	A	C	T
		RK06-113	T	G	T	C	T	C	T	A	G	A	A	C	T
		TR04-88	T	G	T	C	T	C	T	A	G	A	A	C	T
		00MG1626	T	G	T	C	T	C	T	A	G	A	A	C	T
		KG07-77	T	G	T	C	T	C	T	A	G	A	A	C	T
		OH08-124	T	G	T	C	T	C	T	A	G	A	A	C	T
		WM08-215	T	G	T	C	T	C	T	A	G	A	A	C	T
		NWE01	T	G	T	C	T	C	T	A	G	A	A	C	T
		MV09-97	T	G	T	C	T	C	T	A	G	A	A	C	T
		09MG1785A	T	G	T	C	T	C	T	A	G	A	A	C	T
		KM06-227	T	G	T	C	T	C	T	A	G	A	A	C	T
		02MG109	T	G	T	C	T	C	T	A	G	A	A	C	T
		RP08-92	T	G	T	C	T	C	T	A	G	A	G	C	C
		RP08-212	T	G	T	C	T	C	T	A	A	G	A	C	T
		SC08-68	T	G	T	C	T	C	T	A	A	G	A	C	T
		AC08-223	T	G	T	C	T	C	T	A	A	G	A	C	T
		04MG207	T	G	T	C	T	C	C	A	G	A	A	C	T
		TS05-5	T	G	T	C	T	C	C	A	G	A	A	C	T
		MG08-09	T	G	T	C	T	C	C	A	G	A	A	C	T
		04MG2043	T	G	T	C	T	C	C	A	G	A	A	C	T
		TO04-197	T	G	T	C	T	C	C	A	G	A	A	C	T
		AB07-180	T	G	T	C	T	C	C	A	G	A	A	C	T
		AM04-258	T	G	T	C	T	C	C	A	G	A	A	C	T
		IT06-28	T	G	T	C	T	C	C	A	G	A	A	C	T
		MN08-44	T	G	T	C	T	C	C	A	G	A	A	C	T
		JT04-186	T	G	T	C	T	C	C	A	G	A	A	C	T
		BM09-25	T	G	T	C	T	C	C	A	G	A	A	C	T
		BR06-148	T	G	T	C	C	C	T	A	G	A	A	C	T
		SS08-123	T	G	C	C	T	C	T	A	G	A	G	C	C
		04MG2005	T	A	T	C	T	C	T	A	G	A	A	C	T
		04MG2030	T	A	T	C	T	C	T	A	G	A	A	C	T
		07MG351B	T	A	T	C	T	C	T	A	G	A	A	C	T
		CP06-83	T	A	T	C	T	C	T	A	G	A	A	C	T
		DB07-174	T	A	T	C	T	C	T	A	G	A	A	C	T
		DB08-156	T	A	T	C	T	C	T	A	G	A	A	C	T
		KB08-65	T	A	T	C	T	C	T	A	G	A	A	C	T
		LH04-83	T	A	T	C	C	C	T	A	G	A	A	C	T
		NT04-84	T	A	T	C	T	C	T	A	G	A	A	C	T
		MK04-73	T	A	T	C	T	C	T	A	G	A	A	C	T
		03MG365B	T	A	T	C	T	C	T	A	G	A	A	C	T
		DA08-201	T	A	T	C	T	C	T	A	G	A	A	C	T
		EW09-43	T	A	T	C	T	C	T	A	G	A	A	C	T
		AN06-18	T	A	T	C	T	C	T	A	G	A	A	C	T
		ME04-40	T	A	T	C	T	C	T	A	G	A	A	C	T
		RB04-100	T	A	T	C	T	C	T	A	G	A	A	C	T
		VB08-268	T	A	T	C	T	C	T	A	G	A	A	C	T
		LP09-31	T	A	T	C	T	C	T	A	G	A	A	C	T
		JS06-138	T	A	T	C	T	C	T	A	G	A	A	C	T
		08MG1184	T	A	T	C	T	C	T	A	G	A	A	C	T
		WT08-132	T	A	T	C	T	C	T	A	G	A	A	C	T
		JO08-216	T	A	T	C	T	C	T	A	G	A	A	C	T
		WC08-13	T	A	T	C	T	C	T	A	G	A	A	C	T
		BM04-169	T	A	T	C	T	C	T	A	G	A	A	C	T
		LR06-153	T	A	T	C	T	C	T	A	G	A	A	T	T
		MB08-66	T	A	T	C	T	C	T	A	G	A	G	C	T
		RC08-61	T	A	T	C	T	C	T	A	G	A	G	C	C
		RN04-78	T	A	T	C	T	C	T	A	G	A	G	C	C
		TP08-80	T	A	T	C	T	C	C	A	G	A	A	C	T
		WB04-199	T	A	T	C	T	C	C	A	G	A	A	C	T
		DB08-193	T	A	T	C	T	C	C	A	G	A	A	C	T
		DJ08-40	T	A	T	C	T	C	C	A	G	A	A	C	T
		09MG1707A	T	A	T	C	T	C	C	A	G	A	A	C	T
		BC05-33	T	A	T	C	T	C	C	A	G	A	A	C	T
		04MG1207	T	A	T	C	T	C	C	A	G	A	A	C	T
		SR07-13	T	A	T	C	T	C	C	A	G	A	A	C	T
		JH08-220	T	A	T	C	T	C	C	A	G	A	A	C	T
		01MG1188	T	A	T	C	C	C	T	A	G	A	A	C	T
		JR09-45	T	A	T	C	C	C	T	A	G	A	A	C	T
		JD08-139	T	A	T	T	T	C	T	A	G	A	A	C	T
		JM04-62	C	A	T	C	T	C	T	A	G	A	A	C	T
		BM08-127	C	A	T	C	T	C	T	A	G	A	A	C	C
		RR04-35	C	A	T	C	T	C	T	A	G	A	A	C	C
		00MG440													
		02MG2269													
		03MG1940													
		03MG600													
		03MG958													
		DG09-133													
		DR08-152													
		NWF-01													
		PK08-142													
		TC08-55													
		WT08-153													
		04MG1911													
		12846													
		BB08-126													
		CS08-149													
		DS09-125													
		GB08-155													
		GH08-157													
		JH08-137													
		KM08-144													
		MB08-81													
		RN04-78													

*Table 3 shows the mitochondrial haplotypes of all probands involved. The numbers at the top of the table show the position and possible alleles found at that position within the mitochondrial genome. Each nitrogenous base is colour coded so the haplotypes of each proband can be compared at a glance.

Table 3*. Mitochondrial polymorphisms found in each of the deafness probands

FAMILY #	Relationship	PROBAND	MITOCHONDRIAL D LOOP REGION aka VARIABLE REGION aka CONTROL REGION												
			T199C	G203A	T204C	G207A	T217C	C222T	G228A	A235G	T239C	T250C	A257G	A263G	T279C
2010	proband	JT04-49	T	G	T	G	T	C	G	A	T	T	A	G	T
2010	sister	AS07-279	T	G	T	G	T	C	G	A	T	T	A	G	T
2010	sister	EH07-277	T	G	T	G	T	C	G	A	T	T	A	G	T
2010	mother	EK07-281	T	G	T	G	T	C	G	A	T	T	A	G	T
2010	sister	GC08-20	T	G	T	G	T	C	G	A	T	T	A	G	T
2010	paternal uncle	FK07-281	T	G	T	G	T	C	G	A	T	T	A	G	T
2072	proband	CT04-37	T	G	T	G	T	C	G	A	T	T	A	G	T
2072	mother	RT04-38	T	G	T	G	T	C	G	A	T	T	A	G	T
		DJ08-51	T	G	T	G	T	C	G	A	T	T	A	G	T
		EM08-154	T	G	T	G	T	C	G	A	T	T	A	G	T
		GP09-103	T	G	T	G	T	C	G	A	T	T	A	G	T
		RK06-113	T	G	T	G	T	C	G	A	T	T	A	G	T
		TR04-88	T	G	T	G	T	C	G	A	T	T	A	G	T
		00MG1626	T	G	T	G	T	C	G	A	T	T	A	G	T
		KG07-77	T	G	T	G	T	C	G	A	T	T	A	G	T
		OH08-124	T	G	T	G	T	C	G	A	T	T	A	G	T
		WM08-215	T	G	T	G	T	C	G	A	T	T	A	G	T
		NWE01	T	G	T	G	T	C	G	A	T	T	A	G	C
		MV09-97	T	G	T	G	T	C	G	A	T	T	A	G	C
		09MG1785A	T	G	T	G	T	C	A	A	T	T	A	G	T
		KM06-227	T	G	C	A	T	C	G	A	T	T	A	G	T
		02MG109	C	A	C	G	T	C	G	A	T	C	A	G	T
		RP08-92	T	G	C	A	T	C	G	A	T	T	A	G	T
		RP08-212	T	G	T	G	T	C	G	A	T	T	A	G	T
		SC08-68	T	G	T	G	T	C	A	A	T	T	A	G	T
		AC08-223	T	G	T	G	T	T	A	A	T	T	A	G	T
		04MG207	T	G	T	G	T	C	G	A	T	T	A	G	T
		TS05-5	T	G	T	G	T	C	G	A	T	T	A	G	T
		MG08-09	T	G	T	G	T	C	G	A	T	T	A	G	T
		04MG2043	T	G	T	G	C	C	G	A	T	T	A	G	T
		TO04-197	T	G	T	G	C	C	G	A	T	T	A	G	T
		AB07-180	C	G	C	A	T	C	G	A	T	C	A	G	T
		AM04-258	C	G	C	A	T	C	G	A	T	C	A	G	T
		IT06-28	C	G	C	A	T	C	G	A	T	C	A	G	T
		MN08-44	C	G	C	A	T	C	G	A	T	C	A	G	T
		JT04-186	C	G	C	A	T	C	G	A	T	C	A	G	T
		BM09-25	C	G	C	A	T	C	G	A	T	C	A	G	T
		BR06-148	T	G	T	G	T	C	G	A	T	T	G	G	T
		SS08-123	T	G	T	A	T	C	G	A	T	T	A	G	T
		04MG2005	T	G	T	G	T	C	G	A	T	T	A	G	T
		04MG2030	T	G	T	G	T	C	G	A	T	T	A	G	T
		07MG351B	T	G	T	G	T	C	G	A	T	T	A	G	T
		CP06-83	T	G	T	G	T	C	G	A	T	T	A	G	T
		DB07-174	T	G	T	G	T	C	G	A	T	T	A	G	T
		DB08-156	T	G	T	G	T	C	G	A	T	T	A	G	T
		KB08-65	T	G	T	G	T	C	G	A	T	T	A	G	T
		LH04-83	T	G	T	G	T	C	G	A	T	T	A	G	T
		NT04-84	T	G	T	G	T	C	G	A	T	T	A	G	T
		MK04-73	T	G	T	G	T	C	G	A	T	T	A	G	T
		03MG365B	T	G	T	G	T	C	G	A	T	T	A	G	T
		DA08-201	T	G	T	G	T	C	G	A	T	T	A	G	T
		EW09-43	T	G	T	G	T	C	G	A	T	T	A	G	T
		AN06-18	T	G	T	G	T	C	G	A	T	T	A	G	T
		ME04-40	T	G	T	G	T	C	G	A	T	T	A	G	T
		RB04-100	T	G	T	G	T	C	G	A	T	T	A	G	T
		VB08-268	T	G	T	G	T	C	G	A	T	T	A	G	T
		LP09-31	T	G	T	G	T	C	G	A	T	T	A	G	T
		JS06-138	T	G	T	G	T	C	G	A	T	T	A	G	T
		08MG1184	T	G	T	G	T	C	G	A	T	T	A	G	T
		WT08-132	T	G	T	G	T	C	G	A	T	T	A	G	T
		JO08-216	T	G	T	G	T	C	G	A	T	T	A	G	T
		WC08-13	T	G	T	G	T	C	G	A	T	T	A	A	T
		BM04-169	T	G	T	G	T	C	G	A	C	T	A	G	T
		LR06-153	T	G	T	G	T	C	G	A	T	T	A	G	T
		MB08-66	T	G	T	G	T	C	G	A	T	T	A	G	T
		RC08-61	T	G	C	A	T	C	G	A	T	T	A	G	T
		RN04-78	T	G	C	A	T	C	G	A	T	T	A	G	T
		TP08-80	T	G	T	G	T	C	G	A	T	T	A	G	T
		WB04-199	T	G	T	G	T	C	G	A	T	T	A	G	T
		DB08-193	T	G	T	G	T	C	G	A	T	T	A	G	T
		DJ08-40	T	G	T	G	T	C	G	A	T	T	A	G	T
		09MG1707A	T	G	T	G	T	C	G	A	T	T	A	G	T
		BC05-33	T	G	T	G	T	C	G	A	C	T	A	G	T
		04MG1207	T	G	T	G	T	C	G	A	T	T	A	G	T
		SR07-13	T	G	T	G	T	C	G	A	T	T	A	G	T
		JH08-220	T	G	T	G	C	C	G	A	T	T	A	G	T
		01MG1188	T	G	T	G	T	C	G	A	T	T	A	G	T
		JR09-45	T	G	T	G	T	C	G	A	T	T	A	G	T
		JD08-139	T	G	T	G	T	C	G	A	T	T	A	G	T
		JM04-62	T	G	T	G	T	C	G	A	T	T	A	G	T
		BM08-127	T	G	T	G	T	C	G	A	T	T	A	G	T
		RR04-35	T	G	T	G	T	C	G	A	T	T	A	G	T
		00MG440													
		02MG2269													
		03MG1940													
		03MG600												G	T
		03MG958													
		DG09-133												G	T
		DR08-152													
		NWF-01												G	T
		PK08-142													
		TC08-55													
		WT08-153													
		04MG1911												G	T
		12846													
		BB08-126													
		CS08-149													
		DS09-125													
		GB08-155													
		GH08-157													
		JH08-137													
		KM08-144													
		MB08-81													
		RN04-78													

Table 3 continued. Mitochondrial polymorphisms found in each of the deafness probands continued.

			MITOCHONDRIAL D LOOP REGION aka VARIABLE REGION aka CONTROL REGION										
FAMILY #	Relationship	PROBAND	C295T	306 dup C	315 dup C	C325T	455 dup T	C456T	C462T	T477C	T489C	C497T	513_514 del GC
2010	proband	JT04-49	C	C	CC	C	T	C	C	T	T	C	GC
2010	sister	AS07-279	C	C	CC	C	T	C	C	T	T	C	GC
2010	sister	EH07-277	C	C	CC	C	T	C	C	T	T	C	GC
2010	mother	EK07-281	C	C	CC	C	T	C	C	T	T	C	GC
2010	sister	GC08-20	C	C	CC	C	T	C	C	T	T	C	GC
2010	paternal uncle	FK07-281	C	CC	CC	C	T	C	C	T	T	C	GC
2072	proband	CT04-37	C	C	CC	C	T	C	C	T	T	C	GC
2072	mother	RT04-38	C	C	CC	C	T	C	C	T	T	C	GC
		DJ08-51	C	C	CC	C					T	C	GC
		EM08-154	C	CC	CC	C							
		GP09-103	C							T	T	C	GC
		RK06-113	C	CC	CC	C				T	T	C	GC
		TR04-88	C	CC	CC	C	T	C	C	T	T	C	GC
		00MG1626	C	CC	CC	C							
		KG07-77	C	CC	CC	C							
		OH08-124	C	CC	CC	C							
		WM08-215	C	CC	CC	C							
		NWE01	C	C	CC	C	T	C	C	T	T	T	GC
		MV09-97	C	C	CC	C							
		09MG1785A	T	C	CC	C				T	C	C	GC
		KM06-227	C	CC	CC	C							
		02MG109	C	C	CC	C	TT	C	C	T	T	C	GC
		RP08-92	C	C	CC	C			C	T	T	C	GC
		RP08-212	T	CC	CC	C							
		SC08-68	C	C	CC	C	T	C	T	T	C	C	GC
		AC08-223	C	C	CC	C							
		04MG207	C	C	CC	C	T	C	C	T	T	C	GC
		TS05-5	C	C	CC	C							
		MG08-09	C	CC	CC	C							
		04MG2043	C	C	C	C	T	C	C	T	T	T	GC
		TO04-197	C	C	CC	T	T	C	C	T	T	T	GC
		AB07-180	C	C	CC	C							
		AM04-258	C	C	CC	C							
		IT06-28	C	C	CC	C							
		MN08-44	C	C	CC	C						C	GC
		JT04-186	C	C	CC	C							
		BM09-25	C	C	CC	C							
		BR06-148	C	C	CC	C							
		SS08-123	C	CC	CC	C	T	C	C	T	T	C	GC
		04MG2005	C	C	CC	C							
		04MG2030	C	CC	CC	C							
		07MG351B	C	C	CC	C							
		CP06-83	C	C	CC	C							
		DB07-174	C	CC	CC	C							
		DB08-156	C	CC	CC	C				C	T	C	GC
		KB08-65	C	CC	CC	C	T	C	C	T	T	C	GC
		LH04-83	C	CC	CC	C	T	T	C	T	T	C	GC
		NT04-84	C	C	CC	C							
		MK04-73	C	C	CC	C							
		03MG365B	C	C	CC	C							
		DA08-201	C	C	CC	C							
		EW09-43	C	C	CC	C							
		AN06-18	C	C	CC	C							
		ME04-40	C	CC	CC	C							
		RB04-100	C	CC	CC	C							
		VB08-268	C	CC	CC	C							
		LP09-31	C	CC	CC	C							
		JS06-138	C	CC	CC	C							
		08MG1184	C	CC	CC	C							
		WT08-132	C	CC	CC	C							
		JO08-216	C	CC	CC	C							
		WC08-13	C	CC	CC	C			C	T	T	C	GC
		BM04-169	C	CC	CC	C							
		LR06-153	C	C	CC	C					T	C	GC
		MB08-66	C	C	CC	C				T	T	C	GC
		RC08-61	C	C	CC	C				T	T	C	GC
		RN04-78	C	C	CC	C							
		TP08-80	C	CC	CC	C						C	GC
		WB04-199	C	CC	CC	C					T	C	GC
		DB08-193	C	C	CC	C							
		DJ08-40	C	CC	CC	C							GC
		09MG1707A	C	CC	CC	C							GC
		BC05-33	C	CC	CC	C							GC
		04MG1207	C	CC	CC	C	T	C	C	T	T	C	GC
		SR07-13	C	C	CC	C	T	C	T	T	C	C	- -
		JH08-220	C	C	CC	T							
		01MG1188	C	C	CC	C	T	C	C	T	T	C	GC
		JR09-45	C	CC	CC	C							
		JD08-139	C	CC	CC	C				T	T	C	GC
		JM04-62	C	CC	CC	C	T	C	C	T	T	C	GC
		BM08-127	C	CC	CC	C							
		RR04-35	C	CC	CC	C							
		00MG440		CC	CC	C	T	T	C	T	T	C	GC
		02MG2269		C	C	C	T	C	C	T	T	C	GC
		03MG1940		CC	CC	C	T	C	C	T	T	C	GC
		03MG600	C	C	CC	C	T	C	C	T	T	C	GC
		03MG958		C	C	C	T	C	C	T	T	C	GC
		DG09-133	C	CC	CC	C	T	T	C	T	T	C	GC
		DR08-152		C	C	C	T	T	C	T	T	C	GC
		NWF-01	C	C	CC	C	T	C	C	T	T	C	GC
		PK08-142		C	C	C	T	C	T	T	C	C	GC
		TC08-55		C	C	C	T	C	C	T	T	C	GC
		WT08-153		C	C	C	T	C	C	T	T	C	GC
		04MG1911	C	C	CC	C	T	C	C	T	T	C	GC
		12846								C	T	C	GC
		BB08-126											
		CS08-149											GC
		DS09-125								C	T	C	GC
		GB08-155											- -
		GH08-157											GC
		JH08-137						T	C	T	T	C	GC
		KM08-144									T	C	GC
		MB08-81					T	C	C	T	T	T	GC
		RN04-78											

Table 3 continued. Mitochondrial polymorphisms found in each of the deafness probands continued.

			MITOCHONDRIAL D LOOP REGION aka VARIABLE REGION aka CONTROL REGION					TRNF					RNR1	
FAMILY #	Relationship	PROBAND	514_517 dup CA	523_524 del AC	568_569 dup CC	571_573 dup CCC	C575T	T593C	G709A	G719A	A750G	G930A	G951A
2010	proband	JT04-49	- -	AC	C	C	C	T	G	G	G	G	G
2010	sister	AS07-279	- -	AC	C	C	C	T	G	G	G	G	G
2010	sister	EH07-277	- -	AC	C	C	C	T	G	G	G	G	G
2010	mother	EK07-281	- -	AC	C	C	C	T	G	G	G	G	G
2010	sister	GC08-20	- -	AC	C	C	C	T	G	G	G	G	G
2010	paternal uncle	FK07-281	- -	AC	C	C	C	T	G	G	G	G	G
2072	proband	CT04-37	- -	AC	C	C	C	T	G	G	G	G	G
2072	mother	RT04-38	- -	AC	C	C	C	T	G	G	G	G	G
		DJ08-51	- -	AC	C	C	C	T	G	G	G	G	G
		EM08-154		AC	C	CC	C	T	G	G	G	G	G
		GP09-103	- -	AC	C	C	C	T	G	G	G	G	G
		RK06-113	- -	AC	C	C	C	T	G	G	G		
		TR04-88	- -	AC	C	C	C	T	G	G	G	G	G
		00MG1626										G	G
		KG07-77							G	G	G	G	G
		OH08-124								G	G	G	G
		WM08-215											
		NWE01	- -	AC	C	C	C	T	G	G	G	G	G
		MV09-97											
		09MG1785A	- -	AC	C	C	C	T	G	G	G	G	G
		KM06-227							A	G	G	A	G
		02MG109	- -	AC	C	C	C	T	G	G	G	G	G
		RP08-92	- -	AC	C	C	C	T	A	G	G	G	G
		RP08-212											
		SC08-68	- -	AC	C	C	C	T	G	G	G	G	G
		AC08-223											
		04MG207	- -	AC	C	C	C	T	A	G	G	A	G
		TS05-5			C	C	C	T	A	G	G	A	G
		MG08-09								G	G	A	G
		04MG2043	- -	AC	C	C	C	T	G	G	G	G	G
		TO04-197	- -	AC	C	C	C	T	G	G	G		
		AB07-180			C	C	C	T	G	A	G	G	G
		AM04-258			C	CC	C	T	G	A	G		
		IT06-28		AC	C	C	C	T	G	A	G	G	G
		MN08-44	- -	AC	CC	C	C	T	G	A	G	G	G
		JT04-186							G	A	G	G	G
		BM09-25											
		BR06-148										G	G
		SS08-123	- -	AC	C	C	C	T	A	G	G	G	G
		04MG2005			C	C	C	T	G	G	G	G	G
		04MG2030			C	C	C	T	G	G	G	G	G
		07MG351B			C	C	C	T	G	G	G	G	A
		CP06-83							G	G	G		
		DB07-174			C	C	C	T	G	G	G	G	G
		DB08-156	- -	AC	C	C	C	T	G	G	G	G	G
		KB08-65	- -	AC	C	C	C	T	G	G	G	G	G
		LH04-83	- -	AC	C	C	C	C	G	G	G		
		NT04-84											
		MK04-73											
		03MG365B											
		DA08-201											
		EW09-43											
		AN06-18											
		ME04-40									G	G	G
		RB04-100							G	G	G	G	G
		VB08-268							G	G	G	G	G
		LP09-31											
		JS06-138											
		08MG1184											
		WT08-132											
		JO08-216											
		WC08-13	- -	AC	C	C	C	T	G	G	G	G	G
		BM04-169											
		LR06-153	- -	AC	C	C	C	T	G	G	G	G	G
		MB08-66	- -	AC	C	C	C	T	G	G	G	G	G
		RC08-61	- -	AC	C	C	C	T	A	G	G	G	G
		RN04-78											
		TP08-80	- -	AC	C	C	C	T	G	G	G	G	G
		WB04-199	- -	AC	C	C	C	T	G	G	G	G	G
		DB08-193											
		DJ08-40	- -	AC	C	C	C	T	G	G	G	G	G
		09MG1707A	- -	AC	C	C	C	T	G	G	G	G	G
		BC05-33	- -	AC	C	C	C	T	G	G	G	G	G
		04MG1207	- -	- -	C	C	C	T	G	G	G		
		SR07-13	- -	AC	C	C	C	T	G	G	G	G	G
		JH08-220											
		01MG1188	- -	AC	C	C	C	T	G	G	G	G	G
		JR09-45											
		JD08-139	- -	AC	C	C	C	T	G	G	G	G	G
		JM04-62	- -	AC	C	C	C	T	G	G	G		
		BM08-127							G	G	G	G	G
		RR04-35											
		00MG440	- -	AC	C	C	C	T	G	G	G	G	G
		02MG2269	- -	AC	C	C	C	T	G	G	G	G	G
		03MG1940	- -	AC	C	C	C	T	G	G	G	G	G
		03MG600	- -	AC	C	C	C	T	G	G	G		
		03MG958	- -	AC	C	C	C	T	A	G	G	A	G
		DG09-133	- -	AC	C	C	C	T	G	G	G	G	G
		DR08-152	- -	AC	C	C	C	T	A	G	G		
		NWF-01	- -	AC	C	C	C	T	A	G	G	G	G
		PK08-142	- -	AC	C	C	C	T	G	G	G	G	G
		TC08-55	- -	AC	C	C	C	T	G	G	A	G	G
		WT08-153	- -	AC	C	C	C	T	G	G	G	G	G
		04MG1911	- -	AC	C	C	C	T	G	G	G	G	G
		12846	- -	AC	C	C	C	T	G	G	G	G	G
		BB08-126			C	C	C	T	G	G	A	G	G
		CS08-149	- -	AC	C	C	C	T	G	G	G	G	G
		DS09-125	- -	AC	C	C	C	T	G	G	G	G	G
		GB08-155	CACA	AC	C	C	T	T	G	G	G	G	G
		GH08-157	- -	AC	C	C	C	T	G	G	G	G	G
		JH08-137	- -	AC	C	C	C	T	G	G	G	G	G
		KM08-144	- -	AC	C	C	C	T	G	G	G		
		MB08-81	- -	- -	C	C	C	T	G	G	G	G	G
		RN04-78									G	G	G

Table 3 continued. Mitochondrial polymorphisms found in each of the deafness probands continued.

			RNR1					RNR2			
FAMILY #	Relationship	PROBAND	C979T	T1189C	T1243C	A1438G	A1555G	T1700C	G1719A	C1721T	A1763C
2010	proband	JT04-49	C	T	T	G	A	T	G	T	A
2010	sister	AS07-279	C	T	T	G	A	T	G	T	A
2010	sister	EH07-277	C	T	T	G	A	T	G	T	A
2010	mother	EK07-281	C	T	T	G	A	T	G	T	A
2010	sister	GC08-20	C	T	T	G	A	T	G	T	A
2010	paternal uncle	FK07-281	C	T	T	G	A	T	G	C	
2072	proband	CT04-37	C	T	T	G	A	T	G	T	A
2072	mother	RT04-38	C	T	T	G	A	T	G	T	A
		DJ08-51	C	T	T	G	A	T	G	C	A
		EM08-154	C	T	T	G	A	C	G	C	A
		GP09-103	C	T	T	G	A	T	G	C	A
		RK06-113									
		TR04-88	C	T	T	G	A				
		00MG1626	C	T	T	G	A	T	G	C	A
		KG07-77	C	C	T	G	A				
		OH08-124	C	C	T	G	G	T	G	C	A
		WM08-215									
		NWE01	C	C	T	G	A	T	G	C	
		MV09-97									
		09MG1785A	C	T	T	G	A	T	G	C	A
		KM06-227	C	T	T	G	A	T	G	C	A
		02MG109	C	T	T	G	A	T	A	C	A
		RP08-92	C	T	C	G	A	T	G	C	A
		RP08-212									
		SC08-68	C	T	T	G	A	T	G	C	A
		AC08-223									
		04MG207	C	T	T	G	A				
		TS05-5	C	T	T	G	A				
		MG08-09	C	T	T	G	A	T	G	C	A
		04MG2043	C	C	T	G	A				
		TO04-197				G	A	T	G	C	C
		AB07-180	C	T	T	G	A	T	A	C	A
		AM04-258									
		IT06-28	C	T	T	G	A	T	A	C	A
		MN08-44	C	T	T	G	A	T	A	C	A
		JT04-186	C	C	T	G	A				
		BM09-25									
		BR06-148	C	C	T	G	A				
		SS08-123	C	T	C	G	A	T	G	C	A
		04MG2005	C	T	T	G	A				
		04MG2030	C	T	T	G	A	T	G	C	A
		07MG351B	T	T	T	A	A	T	G	C	A
		CP06-83						T	G	C	A
		DB07-174	C	T	T	G	A	T	G	C	A
		DB08-156	C	T	T	G	A	T	G	C	A
		KB08-65	C	T	T	G	A				
		LH04-83				G	A	T	G	C	A
		NT04-84					A	T	G	C	A
		MK04-73									
		03MG365B									
		DA08-201									
		EW09-43									
		AN06-18									
		ME04-40	C	T	T	G	A				
		RB04-100	C	T	T	G	A				
		VB08-268	C	T	T	G	A	T	G	C	A
		LP09-31									
		JS06-138									
		08MG1184									
		WT08-132									
		JO08-216									
		WC08-13	C	T	T	G	A	T	G	C	A
		BM04-169				G	A	T	G	C	C
		LR06-153	C	T	T	G	A	T	G	C	A
		MB08-66	C	T	T	G	A	T	A	C	A
		RC08-61	C	T	C	G	A	T	G	C	A
		RN04-78									
		TP08-80	C	T	T	G	A				
		WB04-199	C	T	T	G	A	T	G	C	A
		DB08-193									
		DJ08-40	C	T	T	A	A				
		09MG1707A	C	T	T	G	A	T	G	C	A
		BC05-33	C	T	T	G	A				
		04MG1207	C	T	T				G	C	A
		SR07-13	C	T	T	G	A	T	G	C	A
		JH08-220									
		01MG1188	C	T	T	G	A	T	G	T	A
		JR09-45									
		JD08-139	C	T	T	G	A	T	G	C	C
		JM04-62						T	G	C	A
		BM08-127	C	T	T	G	A				
		RR04-35									
		00MG440	C	T	T	G	A	T	G	T	A
		02MG2269	C	T	T	G	A	T	A	C	A
		03MG1940	C	T	T	G	A	T	G	C	A
		03MG600									
		03MG958	C	T	T	G	A	T	G	C	A
		DG09-133	C	T	T	G	A	T	G	C	A
		DR08-152				G	A	T	G	C	
		NWF-01	C	T	T	G	A	T	A	C	A
		PK08-142	C	T	T	G	A	T	G	C	A
		TC08-55	C	T	T	A	A	T	G	C	A
		WT08-153	C	T	T	G	A				
		04MG1911	C	T	T	G	A	T	G	C	A
		12846	C	T	T	G	A				
		BB08-126	C	T	T	G	A	T	G	C	A
		CS08-149	C	T	T	G	A	T	G	C	A
		DS09-125	C	T	T	G	A	T	G	C	A
		GB08-155	C	T	T	G	A				
		GH08-157	C	T	T	G	A	T	G	C	A
		JH08-137	C	T	T	G	A				
		KM08-144				G	A	T			
		MB08-81	C	C	T	G	G	T	G	C	A
		RN04-78	C	T	C	G	A				

Table 3 continued. Mitochondrial polymorphisms found in each of the deafness probands continued.

	MITOCHONDRIAL D LOOP REGION aka VARIABLE REGION aka CONTROL REGION																														
DNA	T72C	A73G	T119C	C140T	T146C	C150T	T152C	A153G	G185A	A188G	A189G	C194T	T195C	T199C	G203A	T204C	G207A	T217C	C222T	G228A	A235G	T239C	T250C	A257G	A263G	T279C	C295T	306.dnp C	315.dnp C	C325T	
Control	T	G	T	C	T	C	T	A	G	A	A	C	T	T	G	T	G	T	C	G	A	T	T	A	G	T	C	CC	CC	C	
Control	T	G	T	C	T	C	T	A	G	A	A	C	T	T	G	T	G	T	C	G	A	T	T	A	G	T	C	CC	CC	C	
Control	T	G	T	C	T	C	T	A	G	A	A	C	T	T	G	C	A	T	C	G	A	T	T	A	G	T	C	CC	CC	C	
Control	T	G	T	C	T	C	T	A	G	A	A	C	T	T	G	T	G	T	C	G	A	C	T	A	G	T	C	CC	CC	C	
Control	T	G	T	C	C	C	T	G	G	A	A	C	T	T	G	T	G	T	C	G	G	C	T	A	G	T	C	C	CC	C	
Control	T	A	T	C	T	C	T	A	G	A	A	C	T	T	G	T	G	T	C	G	A	T	T	A	G	T	C	CC	C	C	
Control	T	A	T	C	T	C	C	A	G	A	A	C	T	T	G	T	G	T	C	G	A	T	T	A	G	T	C	CC	CC	C	
Control	T	A	T	C	T	C	T	A	G	A	A	C	T	T	G	T	G	T	C	G	A	T	T	A	G	T	C	CC	CC	C	
Control	T	A	T	C	T	C	T	A	G	A	A	C	T	T	G	T	G	T	C	G	A	T	T	A	G	T	C	CC	CC	C	
Control	T	A	T	C	T	C	T	A	G	A	A	C	T	T	G	T	G	T	C	G	A	T	T	A	G	T	C	C	CC	C	
Control	T	A	T	C	T	C	T	A	G	A	A	C	T	T	G	T	G	T	C	G	A	T	T	A	G	T	C	C	CC	C	
Control	T	A	T	C	T	C	T	A	G	A	A	C	T	T	G	T	G	T	C	G	A	T	T	A	A	T	C	CC	CC	C	
Control	T	A	T	C	C	C	C	A	G	A	A	C	T	T	G	T	G	T	C	G	A	T	T	A	G	T	C	CC	CC	C	

Table 3. Mitochondrial polymorphisms found in controls

Table 4. The number of probands in each category of hearing loss frequency

Category of Hearing Loss	Number of probands with the Specified Type of Hearing Loss
Low Frequency	5
Mid Frequency	2
High Frequency	29
All Frequency	19

Table 5. Mutations found in 2 of the candidate genes targeted by AudioGene.

Gene	Accession number	Mutation	Number of Probands	SNP database frequency	SIFT	Polyphen
Connexin 31	NM_024009	c.109G>A, p. V37M	1	Novel	Deleterious	Damaging
		c.94C>T, p. R32W	6	0.015	Deleterious	Damaging
TMC1	NM_138691	c.421C>T, R141W	4	0.008	Deleterious	Probably Damaging
		c.545G>A, G182D	1	Novel	Tolerated	
		c.1763+3A>G	1	Novel		Probably Damaging

Table 6. A summary of the gene variants found with the proband they were found in and the hearing loss phenotype of the proband. A more detailed table of genes screened and mutations identified is shown in Appendix 5.

Gene Name + Mutation	Proband Family #	Hearing Loss Phenotype
Connexin 30 heterozygous del13S1830 Connexin 26 heterozygous 35delG	2155	Bilateral, profound, sensorineural hearing loss assumed present at birth
Connexin 26 heterozygous 35delG	2197	Bilateral hearing loss, profound in the right ear. Diagnosed at age 29.
Connexin 26 heterozygous F83L	2091	No details available
MTRNR1 A1555G	2112	Severe to profound bilateral hearing loss. Self described as "late deafened"
MTRNR1 A1555G	2144	Self described as "lost hearing during childhood"
mtDNA C150T & T152C	2072	High frequency hearing loss. Age of onset unknown
mtDNA C150T & T152C	2010	High frequency hearing loss. Age of onset unknown
mtDNA G951A	2167	Unilateral, mild to moderately severe hearing loss at mid frequencies. Self reported diagnosis at age 4.
mtDNA G951A	2197	Bilateral hearing loss, profound in the right ear. Diagnosed at age 29.
Connexin 31 V37M	2083	Postlingual hearing loss diagnosed between age 3-25
Connexin 31 R32W	2078	Postlingual hearing loss diagnosed between age 3-25
Connexin 31 R32W	2075	Postlingual hearing loss diagnosed between age 3-25
Connexin 31 R32W	2097	Postlingual hearing loss diagnosed between age 3-25
Connexin 31 R32W	2155	Bilateral, profound, sensorineural hearing loss assumed present at birth
Connexin 31 R32W	2167	Unilateral, mild to moderately severe hearing loss at mid frequencies. Self reported diagnosis at age 4.
Connexin 31 R32W	2156	Adult onset, diagnosed between age 26-50
TMC1 R141W	2010	High frequency hearing loss. Age of onset unknown
TMC1 R141W	2092	Postlingual, diagnosed between the age of 3-25. No hearing in the right ear. Mild to Moderately severe in left ear. Mid to high frequency loss.
TMC1 R141W	2177	Right borderline mild low frequency sloping to moderately severe high frequency sensorineural hearing loss which is reported to have started during childhood. The left ear is affected by left mild high frequency sensorineural hearing loss
TMC1 R141W	2146	right severe to profound reverse slope sensorineural hearing loss. In his left ear he experiences steep high frequency sensorineural hearing loss
TMC1 G182D	2124	Postlingual hearing loss diagnosed between ages 3-25
TMC1 c.1763+3 A>G	2065	Bilateral symmetrical sensorineural mild to moderate hearing loss at low frequencies, severe hearing loss at high frequencies.

4. Discussion

The purpose of this study was to determine the genetic etiology of probands affected by nonsyndromic hereditary hearing loss. The probands in this study are all originally from the island of Newfoundland, a founder population. An island with a founder population is an ideal place for genetic studies. This study focused on families with a history of hearing loss. Previous studies on hearing loss in the Newfoundland population found causative mutations in *WFS1¸ TMPRSS3*, and *PCDH15*. These mutations, however, were not found in the families involved in this project. Mutations were identified in the mitochondrial gene, *MTRNR1,* as well as nuclear genes, *Cx26*, *Cx30*, *Cx31*, and *TMC1*.

4.1 Screening Probands for Mutations previously found in the NL Population

The initial step of this study was to screen all probands for mutations in genes previously found to cause hearing loss in the Newfoundland population. In 2001, A p. 2146 G>A mutation that was causing a dominant form of inherited progressive deafness was identified in the *WFS1* gene (Young *et al.*). Two recessive mutations in *TMPRSS3* were found to underlie hearing loss in a six-generation family from the south coast of Newfoundland (Ahmed *et al.*, 2004), and Doucette *et al.* (2009) identified a novel mutation in *PCDH15* causing profound hearing loss in another south coast family. All 110 probands were screened for these mutations, however all individuals were negative. This is interesting given that these mutations were deemed founder mutations. It would

be expected that the mutations would show up while screening a group of individuals with hearing loss from the island. It is possible that the sample considered in this study was not large enough. In future studies, more hearing loss patients should be recruited to determine the accuracy of describing the NL mutations (in *WFS1*, *TMRSS3*, and *PCDH15)* as founder mutations.

4.2 Proband from family 2155 with Hearing Loss Caused by Mutations in connexin 26 & Connexin 30

Mutations in genes that are located in the *DFNB1* locus, connexin 26 (*Cx26*) and connexin 30 (*Cx30*), are the cause of 30-50% of this type of hearing loss (Bhalla *et al.*, 2009). I therefore decided to screen all probands for mutations in these genes once the NL mutations were ruled out.

The proband from family 2155 was found to carry a heterozygous c.35delG mutation in exon 2 of *Cx26* as well as a heterozygous 342 kb deletion in *Cx30.* These genes encode connexin proteins. If only one of the genes has a mutation (i.e. a heterozygous mutation), there is enough functional protein produced to allow the gap junctions to function properly, therefore there must be a homozygous mutation in either *Cx26* or *Cx30*, or one mutation in each of the genes, to cause hearing loss (Smith *et al.*, 2014). In the case of the proband from family 2155 there is a heterozygous 35delG mutation in connexin 26 and a heterozygous 342 kb deletion in connexin 30 together causing hearing loss through digenic inheritance. The c.35delG mutation in connexin 26 is a known mutation first found in hearing impaired Mediterranean patients in 1997 by Zelante *et al*. This particular mutation is common in Caucasian populations and is the

result of a worldwide founder effect (Laer *et al.,* 2001). The deletion of a guanine at position 35 in exon 2 of connexin 26 is a frameshift mutation that leads to premature chain termination causing the production of a truncated, dysfunctional connexin 26 protein. The large deletion in connexin 30, given the name Δ(*GJB6*-D13S1830), truncates the connexin 30 protein, in addition, the deleted portion contains a *cis* regulatory element, that regulates the production of the connexin 26 protein (Rodriguez-Paris *et al*., 2011). With the deletion of this regulatory element in the connexin 30 gene, the expression of the connexin 26 protein is terminated, therefore no connexin 26 protein is produced (del Castillo *et al.*, 2002). In European populations this deletion is the second most frequent mutation related to autosomal recessive hearing loss (Esteves *et al.*, 2013). Thus, the presence of both mutations causes the hearing loss that affects the proband from family 2155.

Digenic inheritance of the 35delG mutation in connexin 26 and Δ(*GJB6*-D13S1830) in connexin 30 has been shown to cause variable hearing loss. In one study published in 2004 (Bolz *et al.*) the severity of hearing loss due to this digenic inheritance was significantly different between the two families included in the study, with one family affected by profound hearing impairment and the other affected by moderate/severe hearing loss. The reason for the difference in phenotype in this case is that homozygous mutations in both *Cx26* and *Cx30* are found in affected members of the families. Therefore the severity of the phenotype depends on which mutations the individual carries. Within one family there can be different combinations of the mutations. As mentioned in the report, one patient has a less severe hearing loss, which

could be caused by a modifier element that is upregulating connexin 26 or connexin 30. The hearing loss is generally more severe if the patient has a homozygous mutation in connexin 26 or connexin 30 (Bolz *et al.*, 2004; Erbe *et al*., 2004; Marlin *et al.*, 2005). However, a different study found probands with del(*GJB6*-D13S1830)/c.35delG presented with a more severe hearing impairment (Batissoco *et al.*, 2009). Another study published in 2013, by Esteves *et al.,* states that patterns of hearing loss caused by mutations in the connexin 26 gene have not been established, and there is no consistent model of the phenotype caused by malfunctioning or nonfunctioning connexin 26 proteins. Therefore the phenotype caused by the digenic inheritance of the 35delG mutation and Δ(*GJB6*-D13S1830) in connexin 30 is still uncertain. The severity of hearing loss caused by mutations in *Cx26* and *Cx30* is highly variable, so it is difficult to associate these mutations with a specific severity (Cama *et al.,* 2009; Esteves *et al.*, 2013).

The proband from family 2155 is a 25-year-old woman who was diagnosed with bilateral, profound, sensorineural hearing loss at the age of 18 months. It was recognized at the age of 9 months that the proband had no speech development, and it is assumed that she was hearing impaired since birth. As mentioned, the hearing loss phenotype caused by *Cx26* and *Cx30* mutations is variable, and the hearing loss presented by this proband is of similar clinical presentation found in previous studies (Cama *et al.,* 2009; Esteves *et al.*, 2013). It is also a similar clinical presentation given for *DFNB1* hearing loss, which is congenital, non-progressive, mild-to-profound sensorineural hearing impairment, with no other medical findings (Retrieved from www.ncbi.nlm.nih.gov

/sites/GeneTests/ review?db=GeneTests on July 15, 2011). As both genes are found within the *DFNB1* locus, the mutations found in this proband justify the hearing loss profile.

4.3 Proband from family 2197 found to have a heterozygous 35delG mutation in *Cx26*

A heterozygous 35delG mutation was found in exon 2 of *Cx26* in the proband from family 2197 (Fig.3.6). This mutation alone is not the cause of hearing loss in this individual, as the 35delG mutation in *Cx26* has never been reported to cause hearing loss in the heterozygous state (Kaskalan *et al.*, 2014). I screened *Cx30* in this individual, however no mutations were detected. This proband was also included in all further gene screening, which resulted in no additional mutation detection. This individual has a bilateral asymmetric hearing loss that was diagnosed at age 29 and is more profound in the right ear. There is no medical information available for this person's family; therefore it is difficult to determine if the single deafness allele was inherited or has arisen de novo.

4.4 A heterozygous F83L variant in *Cx26* exon 2 in the proband from family 2091

The proband from family 2091 was found to have a heterozygous F83L variant in *Cx26*, exon 2. Using four bioinformatics programs to predict the pathogenicity of this variant, all predict this missense variant to be benign (Table 1). As well the Weblogo (Fig.3.10) shows that the phenylalanine at position 83 is not well conserved. The variant has also been reported in the literature to be a polymorphism (Ross *et al.*, 2007; Minárik

et al., 2012). I concluded that this variant is probably not responsible for causing hearing loss in the proband. There was no audiogram available for this patient so it was difficult to choose candidate genes based on audioprofiling. Obtaining a more extensive medical background for the individual and the family would help for future studies.

4.5 Variants in the Mitochondrial Genome

Mutations in two mitochondrial genes (*MTRNR1* and *MTTS1*) have been identified to cause hearing loss. The mitochondrial genome is exceptionally polymorphic and varies greatly among individuals. Sometimes these variants are disease causing, such as the case with A1555G in *MTRNR1*, and at other times there are variants commonly found in the general population (Kazuno *et al.*, 2006). I chose to screen for mutations in the mitochondrial genes following the screening for *Cx26* and *Cx30* mutations as mutations in the mitochondrial genome are one of the most common cause of nonsyndromic hearing loss (Guo, 2008). I screened *MTRNR1* and *MTTS1* in all unsolved probands. Seven polymorphisms were found and two probands were found to have the A1555G mutation in *MTRNR1*.

4.6 Probands from families 2112 and 2144 Hearing Loss caused by a mutation in *MTRNR1*

The probands from families 2112 and 2144 were found to have a homoplasmic A1555G mutation in the *MTRNR1* gene. *MTRNR1* is a mitochondrial gene that codes for the 952 nucleotide long 12S ribosomal RNA (rRNA). The substitution of a guanine for an adenosine at position 1555 in the mitochondrial genome within the *MTRNR1* gene is

known to cause aminoglycoside induced hearing loss. Aminoglycosides are a group of antibiotics that rid the body of harmful bacteria by binding to their 16S rRNA in the 30S ribosomal subunit and inhibiting protein synthesis. The A1555G mutation causes the 12S rRNA to resemble the bacterial 30S rRNA, thus when an individual with the A1555G mutation is given aminoglycoside treatment, the aminoglycosides will inhibit protein synthesis within their mitochondria (Guan, 2011).

The proband from family 2112 has been diagnosed with severe to profound bilateral hearing loss. She self-described being late deafened, having lost her hearing after an intravenous antibiotic treatment for pneumonia (which the individual reported as penicillin, not an aminoglycoside). The proband reported that her mother was affected with hearing loss after she was treated for tuberculosis in the 1950s. This medical information possibly links the hearing loss to the mitochondrial A1555G mutation in the proband, as well as the proband's mother, obtaining DNA from the proband's mother would help confirm this. In the 1950s, tuberculosis was treated using streptomycin, an aminoglycoside (Mitchison, 2005). Individuals who have the A1555G mutation often develop hearing loss following the administration of aminoglycosides (Guan, 2011). The proband of family 2112 has three siblings, all male; one affected by progressive bilateral hearing loss. However, DNA was not available for these individuals.

The hearing loss in family 2112 is likely a case where both genetics and environment come into play. The A1555G mutation is not known to cause hearing loss on its own, but can predispose an individual to lose hearing when exposed to aminoglycosides. Cases such as this one show that a combination of genetic and

environmental factors can be the cause of a condition. It is common to attribute hearing loss to an environmental cause, such as a head injury, medication, infection, or noise pollution. However, the appearance of hearing loss after such occurrences could be purely coincidental, or predisposed by a genetic cause. Accepting that an environmental occurrence is the only reason for an individual's hearing loss and failing to further investigate the reason a person develops the disorder can result in the omission of an underlying genetic cause.

The proband from family 2144, with *MTRNR1* A1555G, reports that her hearing loss began during childhood after an airplane flight and an ear infection and it has been getting progressively worse since then. She has had repeated ear infections as well as premature graying, a minor diagnostic criterion for syndromic deafness known as Waardenburg Syndrome (Nayak and Isaacson, 2003). As there is little information pertaining to this probands medical history and family history it is difficult to make connections between her hearing loss and genotype. Again, obtaining more information from the families should be looked into as further studies would help shed more light on this mutation and the environmental triggers of genetic hearing loss.

4.7 A G951A variant in *MTRNR1* found in two probands from families 2167 and 2197

A G951A variant in *MTRNR1* was found in the proband from family 2167 (Fig.3.11) and in the proband from family 2197, who also has a heterozygous 35delG mutation in connexin 26 as previously discussed (Fig.3.6). It cannot be concluded that this mitochondrial variant is the cause of the hearing loss expressed by these individuals. Elstner *et al.* (2008) identified this variant in the 12S rRNA of 66 patients with bilateral

vestibulopathy (damage to the inner ear(s) causing dizziness, imbalance, and vision disturbances) and also in 155 healthy controls. None of the individuals sequenced had mutations in the mitochondrial genes that are known to cause hearing loss (A1555G or C1494T). They did find four mutations with putative pathogenic effects, T669C, C960del, C960ins, and T961G. They also found five frequent polymorphisms: G709A, G930A, T1189C, T1243C, G1438A and seven of what they called "rare homoplasmic changes": T669C, T721C, G951A, C960del, C960ins, T961G, and G1007A, when they compared the 12S rRNA sequence to the Cambridge reference sequence (Elstner *et al.*, 2008). G951A was not found in 155 healthy patients; however they reported that no assumptions could be made about this variant and concluded that none of the variants they found had proven pathogenicity (Elstner *et al.*, 2008). The report by Elstner *et al.* (2008) was the only evidence of G951A in the literature and it provided no information to the pathogenicity of G951A. Given the limited evidence, it can only be concluded that the G951A variant is of unknown pathogenicity. Further investigation of the genotype is required to determine the effect of the G951A variant.

The proband from family 2167 (Fig.3.11) was diagnosed with unilateral mild to moderately severe sensorineural hearing loss in his left ear that presents in a "cookie bite" audiometric configuration (severe hearing loss in the mid frequencies with mild to no hearing loss in the higher and lower frequencies). He self reports that the hearing loss is progressing and that he is also affected by premature graying (before the age of 30) as well as kidney problems. He also reports that he had multiple left sided ear infections throughout his life and his hearing loss was diagnosed when he had a preschool

examination at age four. There are three distant relatives that have been diagnosed as deaf. Acquiring their DNA may not help to determine the pathogenicity of the G951A mutation because all relatives diagnosed as deaf are on the paternal side of the family, however, if the proband's mother and father are descendants of the same maternal ancestor it is possible that both the mother and father have this mutation. It would be beneficial to obtain more information on the maternal side of the family to help solve the questions of pathogenicity that surround the G951A variant.

The proband from family 2197 has a bilateral asymmetric hearing loss that was diagnosed at age 29 and is more profound in the right ear. There was no available audiogram; therefore it was difficult to choose candidate genes based on audioprofiling. The proband had an affected father as well as two affected uncles. The paternal grandmother was also affected, suggesting that hearing loss was segregating as a dominant trait on the paternal side This proband also has a heterozygous 35delG mutation in connexin 26. It is possible that an interaction between connexin 26 and mitochondrial mutations may be having an effect in this individual. However, because no conclusion can be drawn as to whether or not the G951A mutation is pathogenic, then it is difficult to say it is affecting the wild type connexin 26 gene. A literature search revealed no previous studies that discussed the G951A mutation in connection with a connexin 26 mutation. Future studies should look closely at the interaction between *MTRNR1* and *Cx26*, it is interesting as mutations in both cause hearing loss.

4.8 *MTRNR1* common variants

There are many common variants in mitochondrial DNA that are not associated with disease, including six such variants in *MTRNR1* (Retrieved from http://www.mitomap.org/MITOMAP on May 31, 2011): G709A, A750G, A751G, G930A, T1189C, and G1438A. Five of these variants are previously reported as found at significant frequencies across several ethnic groups. Because they are at a frequency greater than 1% in the general population, these mitochondrial variants are considered common variants (Lu *et al.,* 2010; Rydzanicz *et al.,* 2010; Elstner *et al.,* 2008; Li *et al.,* 2004). In 2004 Li *et al.*, in their study of 164 Caucasian subjects under 19 years of age who were diagnosed with nonsyndromic sensorineural deafness, reported that A751G was a novel polymorphism in the Caucasian population. In 2009, a Polish group, Rydzanicz *et al.*, found this sequence variant and submitted it to MITOMAP (reference #20071219002) as it had not been reported on MITOMAP at that time. They called A751G a nucleotide change, but made no other comment about its pathogenicity. No variants, pathogenic or non-pathogenic, were found in *MTTS1*.

The associations made between mitochondrial variants and disease are dependent on population genetics and because of the variability of the mitochondrial genome it is often difficult to make conclusions about the pathogenicity of a polymorphism (Kazuno *et al.*, 2006). The variants found in this study have been published multiple times; therefore it is likely that the pathogenicity assigned to each variant is correct.

4.9 Specificity of Mitochondrial Primer Binding Sites

The proband from family 2072 appeared to have a mitochondrial deletion, however, following several attempts using different primer sequences a high quality sequence was finally obtained and sequence analysis showed that two SNPs (C150T, and T152C) were present in the mitochondrial DNA of the proband, therefore the hypothesis that the primer is hybridizing at a point 349 bp away from the intended primer hybridization site is most likely correct. To further support this hypothesis, specificity at the 3' end of a primer to the DNA sequence is extremely important as primers that have poor specificity in this region usually produce undesirable amplicons (Dieffenbach, T, & G, 1993). In order to have a successful PCR it is important to have the correct placement of the 3' end of a primer so that perfect base-pairing between the 3' end of the primer and the template DNA is necessary and minimal mismatch should be present (Dieffenbach *et al*., 1993). Therefore, the presence of these SNPs where the 3' end of the primer should hybridize decreases the specificity of the primer and causes the primer to hybridize at a position 349 base pairs away that matches the last six base pairs of the 3' end of the primer.

4.10 Matching Mitochondrial Haplotypes

Following the discovery that the probands of family 2010 and 2072 has the same SNPs in their mitochondrial DNA, I thought it would be interesting to see if their mitochondrial haplotype was identical. No other families had the same combination of SNPs. This suggests that these families may be related through the maternal line, which is

likely, as they appear to have the same haplotype and they come from the same area of the island of Newfoundland. Upon the discovery of the similarity of the SNPs found in these two families, the audiograms were analyzed and it was noticed that the audiograms that were available for the family members of 2010 and 2072 showed very similar hearing loss patterns (Fig.3.21). The original SNPs found in these families (C150T and T152C) could play a role in the hearing loss that the affected members have, or they might just indicate that these families are related through the maternal line, and have the same type of hearing loss caused by possible shared mutations. The audiograms and family history of all other probands showing similar mitochondrial haplotypes should be analyzed to see if they have similar hearing loss patterns.

4.11 Connexin 31 Variants

Mutations in connexin 31 have been shown to cause both dominant and recessive hearing loss (Liu *et al.*, 2000). Two mutations have been reported to cause dominant hearing loss, R180X and E183K, and two mutations have been found to cause recessive hearing loss, 141del_Ile, and I141V (Retrieved from http://hereditaryhearingloss.org on May 12, 2014). Few publications regarding this connection exist in the literature (Retrieved from davinci.crg.es/deafness/ on May 12, 2014). The recessive mutations were found in a study of 25 Chinese families with apparent recessive hearing loss. The study, conducted by Liu *et al.* (2000), found a sibling pair, with severe hearing loss at all frequencies, and a single proband with moderate hearing loss at all frequencies, to be compound heterozygotes for the 141delIle and I141V mutations, neither of the parents were affected. In the case of this study, the probands screened for connexin 31 mutations

had high frequency hearing loss, which does not fit the phenotype for recessive connexin 31 hearing loss found in the study by Liu *et al.* (2000). However, the gene was suggested as a candidate following the audioprofile investigation, and so screening went ahead.

Connexin 31 was screened in 29 probands with high frequency hearing loss and two variants were discovered in 7 probands. The variant c.109G>A, p.V37M was found in 1 proband; and c.94C>T, p.R32W was found in 6 probands.

The variant c.109G>A, p.V37M was found in family 2083 (Fig.3.26). The proband from this family has postlingual hearing loss, which was reported to have occurred between the ages of 3 and 25. Xia *et al.* (1998) report that autosomal dominant deafness caused by a c.538C>T in patients who lost their hearing in late adulthood; this onset is dissimilar to the onset in this proband. In order to reach a conclusion about the pathogenicity of this variant, a more detailed medical history of the proband is required as well as DNA from the probands family members.

The mutation c.94C>T, p.R32W was found in six families. The families with this *Cx31* mutation are: family 2155(Fig.3.5), family 2167 (Fig. 3.11), family 2078 (Fig. 3.22), family 2075 (Fig. 3.23), family 2097 (Fig. 3.24), and family 2156 (Fig 3.25). The proband from family 2097 has postlingual hearing loss diagnosed between the ages of 3 and 25; this proband also has a heterozygous connexin 30 deletion Δ (*GJB6*-D13S1830). While it has not yet been determined that these two mutations cause hearing loss when inherited together, it is worth looking into for future studies. The proband from family 2155 has been previously described as this proband also has been found to have digenic

inheritance of the 35delG mutation in connexin 26 and Δ(*GJB6*-D13S1830) in connexin 30. This proband is also homozygous for R32W in connexin 31. These mutations have not been found inherited together, and there is no published evidence of these two genes interacting, but it is possible that having mutation in three of these connexin genes could increase the severity of the hearing loss and is worth looking into in future studies. The proband from family 2075 has a history of postlingual hearing loss diagnosed between the ages of 3 and 35. The proband from family 2156 also has adult onset hearing loss, diagnosed between 26-50 years old. The proband from family 2078 (Fig.3.22) has postlingual hearing loss. The proband from family 2167 has been previously mentioned as he has a variant in the mitochondrial DNA, he was diagnosed between the ages of 3 and 25, he hears well at low and high frequencies but not at middle frequencies. This is called a "cookie bite" curve on an audiogram because the curve is high on both ends but low in the middle and appears as if there is a bite taken out of it. All of these probands present with different types of hearing loss, and given the variability of hearing loss caused by mutations in connexin 31 (OMIM) you cannot rule out either of them based on phenotype. However, the pathogenicity of R32W has been questioned in the literature. In one study, R32W was found in hearing loss patients, but was also found in 18 percent of the control population (Lopez-Bigas *et al.*, 2000). Rouan *et al.* (2003) used HeLa cells to test *Cx31* function when the R32W variant was present, and found no deviations in expression level, concluding that it is an inconsequential polymorphism.

It is interesting that these variants appear to segregate with hearing loss in these families however a more detailed segregation analysis is required to confirm

pathogenicity (Fig.3.27, 3.28, 3.29, 3.30). Therefore in future studies segregation analysis should be performed. In addition to segregation analysis it would be extremely beneficial to obtain a more detailed family and medical history for each of these probands, given that the family would be willing to divulge that information.

4.12 *TMC1* Variants

Mutations in the *TMC1* gene are known to cause nonsyndromic autosomal dominant and autosomal recessive hearing loss (Hereditary Hearing Loss Homepage, OMIM, www.omim.org). Almost all reported cases of *TMC1* hearing loss report pre-lingual severe to profound hearing loss (OMIM; Gao *et al.*, 2013). *TMC1* is a second gene that was suggested as a candidate gene for hearing loss probands following the audioprofiling investigation. Three mutations were found in *TMC1*: c.545G>A:p.G182D in the proband from family 2124, c.1763+3A>G in the proband from family 2065 (Fig. 3.32), and R141W in the proband from families 2010 (Fig. 3.20), 2146 (Fig. 3.33), 2177 (Fig.3.34), and 2092 (Fig.3.35). All of these probands are affected by post lingual hearing loss. There is no additional information on the proband from family 2124 (Fig. 3.31).

The proband from family 2065, with the intronic mutation, c.1763+3A>G., was born in 1996 and has bilateral symmetrical sensorineural mild to moderate hearing loss in the low frequencies (250-750 Hz), and severe hearing loss in high frequencies (1000-8000Hz) that progressed rapidly in the first decade of his life. Mutations in *TMC1* causing nonsyndromic hearing loss have been found more commonly in Pakistani families (OMIM), however one study has found *TMC1* hearing loss in a North American

Caucasian family (Kitajiri *et al.*, 2007). The affected members from this family had postlingual, progressive sensorineural hearing loss, which presented in the second decade of life, starting in the high frequencies. The mutation found in this family was a missense substitution c.G1714C, p.D572H, and falls within a large cytoplasmic loop region of the *TMC1* protein (Kitajiri *et al.*, 2007; Labay *et al.*, 2010). While this mutation was not found in the proband from family 2065, the region of *TMC1* that is mutated in the proband lies within an intron of close proximity. Analysis of this mutation and of the proband's family is required to determine the pathogenicity of the c.1763+3A>G variant.

The proband from family 2010, with the R141W mutation, was born in 1965 and has normal bilateral hearing at 250-500 Hz (low frequency), with a precipitous slope over 500 Hz where the hearing loss (bilateral and sensorineural) becomes moderately severe to profound in high frequencies. The proband has two male relatives, who report having hearing loss by age five, one of which had normal hearing at birth and in the other, the age of onset is unknown. This proband also has more severe hearing loss in the higher frequencies which is also the case with the *TMC1* hearing loss in the North American family reported by Kitajari *et al.* (2007). Acquiring DNA from the proband's family members is essential in determining the pathogenicity of this variant.

The proband from family 2092 was born in 1935. She has flat line hearing loss (i.e. absolutely no sense of hearing) in her right ear. In her left ear she has mild sloping to moderately severe sensorineural hearing loss in the mid to high frequencies. Audiograms of other relatives show symmetrical bilateral loss, the same as this proband is experiencing in her left ear. Once again, we see hearing loss affecting the high

frequencies, which appears to be the case in the only Caucasian family identified to have hearing loss caused by a mutated *TMC1* gene (Kitajiri *et al.*, 2007). There is no information as to the rate of progression of hearing loss for this individual. To determine the effect of the mutation, DNA should be acquired from the available family members.

The proband from family 2146, born in 1958, is affected by right severe to profound reverse slope sensorineural hearing loss. In his left ear he experiences steep high frequency sensorineural hearing loss above 2000 Hz. He reports normal hearing bilaterally at age 8-10 years and an onset of Meniere's disease (a disease causing sporadic episodes of vertigo, sensorineural hearing loss, tinnitus and ear pressure ;OMIM, www.omim.org) symptoms in his right ear at age 37. He displays hearing loss, vertigo attacks, tinnitus and ear fullness, all symptoms of classic Meniere's disease with classic progression in the right ear over 15 years with increasing hearing loss with every attack, and partial recovery of hearing to the previous level between attacks. The high frequency hearing loss appears to be common among all individuals found to have mutations in *TMC1*. Because this type of hearing loss was displayed in the Caucausian family with *TMC1* hearing loss (Kitajiri *et al.*, 2007) this could be an indication that it may be causing hearing loss in these individuals. There has been no evidence of *TMC1* mutations associated with Meniere's disease.

Lastly, the proband from family 2177 was born in 1961 and has right borderline mild low frequency sloping to moderately severe high frequency sensorineural hearing loss, which is reported to have started during childhood. The left ear is affected by left mild high frequency sensorineural hearing loss at 8000 Hz. Episodes of vertigo prompted

a tentative diagnosis of Meniere's disease, but it is not a classic presentation. The proband's aunt and brother reported having a diagnosis of Meniere's disease with asymmetric hearing loss. This individual also displays more severe hearing loss in the high frequencies, which was seen in all other probands with *TMC1* mutations. Both bilateral hearing loss and asymmetrical hearing loss has been displayed in the probands, and the *TMC1* mutations causing hearing loss do not seem to specifically cause one or the other (Kitajiri *et al.*, 2007). This proband has also been diagnosed with Meniere's disease, which is interesting and worth looking into, if it were found to be the result of the *TMC1* mutation, it would be the first known case.

Like the probands with connexin 31 mutations, it will be necessary to perform segregation analysis to verify the pathogenicity of the variants found. Also, retrieving a more detailed family history may also present clues as to what type of hearing loss affects these probands. It would be valuable to have an extensive family history and medical history on each of these individuals.

4.13 Conclusion

Overall the study resolved the etiology for four hearing loss probands out of 110 that were screened. One proband has hearing loss caused by a homozygous 35delG mutation in connexin 26, another proband has hearing loss caused by the digenic inheritance of the 35delG mutation in connexin 26 and del13S1830 in connexin 30. Two probands have the A1555G mutation in the *MTRNR1* gene of the mitochondrial genome.

Other probands (n=7) have variations in connexin 31 and 6 others have variations in *TMC1*, yet these cannot be considered resolved because segregation analysis has not yet been performed to confirm the pathogenicity of the variants.

In addition, this study found that none of the probands recruited for this project had mutations previously found to cause hearing loss in the Newfoundland population (*TMPRSS3*, *PCDH15*, and *WFS1*). Inviting questions as to whether or not these are actually founder mutations. Also of interest are the similarities between mitochondrial haplotypes of several of the probands, especially those from family 2072 and 2010. The affected members of this family appear to have the same type of hearing loss, however an underlying genetic cause could not be identified. This study has brought forward mutations in several genes (*Cx31*, *TMC1*, *MTRNR1*) that were not previously found in the Newfoundland population. These findings create many opportunities to further explore the genetic etiology of hearing loss in this province.

Determining the underlying cause of hearing loss in Newfoundland and Labrador will improve the province's screening strategies so that this disorder can be identified as early as possible. Recognizing hearing loss early in life can positively impact a child and the ability to learn communication skills and participate in a school environment. Children who are identified as hearing impaired can be taught to communicate in a specialized manner or can also benefit from certain types of hearing devices, such as hearing aids or cochlear implants. In addition to this, knowing the cause of deafness can offer relief to families who can then receive support from genetic counselors and understand the risk of passing the disorder on to their children.

5. Future Directions

This study opens up many possibilities for future projects. First of all it is necessary to perform segregation analysis on the variants of connexin 31 found in 7 of the probands and the variants of *TMC1* found in 6 of the probands, and to perform exome sequencing (sequencing only the coding regions) on the unsolved probands with the intent to find known and novel mutations. Secondly, it would be beneficially to recruit more family members and collect familial DNA for those probands that were lacking family medical history or for which there was no family DNA available (family 2197). Having the information and resources to further studies into the cause of their hearing loss may aid in finding a cause. A study could also be built around the mitochondrial haplotypes that were presented in this project. These haplotypes can be used to identify the relationships and origins of families. Linking haplotypes to phenotypes could potentially solve several families. More specifically it would be interesting to map the lineage of families 2072 and 2010 since this study discovered several similarities among the members of these families affected by hearing loss. Lastly because this study dealt with a small population of deaf probands a recruitment project could bring in more families affected by this disorder. Screening the families would give a more in depth look at mutations previously identified as founder mutations within the Newfoundland population.

While this study has significantly added to the research on hearing loss genetics it has also opened the door for many more projects to be explored. With the quick advancement of DNA sequencing technology, as well improvements for screening

deafness, hopefully the studies can be carried out and conclusions can be drawn from the information uncovered in this research project.

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Table 1. The Forward and Reverse primers and PCR conditions for each gene.

Gene	Forward Primer	Reverse Primer	PCR Conditions
GJB2 Exon 1	TCCGTAACTTTCCCAGTCTCCGAGGG	CCCAAGGACGTGTGTTGG TCCAGCC	TD-56: 94 ºC for 5s, 94ºC for 30s, 66 ºC for 30s, 72 ºC for 30s, for 2 cycles. 94ºC
GJB2 Exon 2	TGCTTACCCAGACTCAGAGAA	CGACTGAGCCTTGACAGCTGA	Connexin: 94 ºC for 4s,94 ºC for 1s, 60 ºC for 1s, 72 ºC for 1s for 35 cycles.72
GJB6 c	AGGGATAAACCAGCGCAATG	AGCACAACTCTGCCACGTTA	Connexin: 94 ºC for 4s,94 ºC for 1s, 60 ºC for 1s, 72 ºC for 1s for 35 cycles.72 ºC for 5s, soak cycle at 4 ºC
GJB6 d	AGTGCTGGGATTACAGGTAC	AGCACAACTCTGCCACGTTA	
TMPRSS3 Exon 4	GACAGGGACGCAATTTCAATA	TTCAATCCCAGCTGAAGACA	TD-60: 95ºC for 2 minutes, 10 cycles of 94ºC for 45s, 70ºC for 1 min, and 72ºC for 1 min, 35 cycles of 94ºC for 45s, 60ºC for 45s, and 72ºC for 1 min,72ºC for 10 min
TMPRSS3 Exon 8	TAGAGCTGCTGTGAGCTCTG	AGACTCCTCTCCAACTGTAC	
PCDH15 Exon 13	GCCAACCTTGTCTTCATCGT	GCAACAGAGCGAGACTCCAT	TD-54: 94 ºC - 5 minutes, 5 cycles of 94ºC-30s, 64 ºC-30s, 72 º-30s, then 30 cycles of 94ºC-30s, 54 ºC-30s, 72 ºC-30s
WFS1 Exon 8	GCCAACCTTGTCTTCATCGT	GCAACAGAGCGAGACTCCAT	TD-56: 94 ºC for 5s, 94ºC for 30s, 66 ºC for 30s, 72 ºC for 30s, for 2 cycles. 94ºC for 30s, 56 ºC for 30s, 72 ºC for 30s for
MTRNR1 Section 1	TTGATTCCTGCCTCATCCT	CTTTACGCCGGCTTCTATTG	TD-54: 94 ºC - 5 minutes, 5 cycles of 94ºC-30s, 64 ºC-30s, 72 º-30s, then 30 cycles of 94ºC-30s, 54 ºC-30s, 72 ºC-30s and finally 72ºC for 7 minutes and a soak cycle at 4 ºC
MTRNR1 Section 2	AATGTTTAGACGGGCTCACA	CCTGTTCAACTAAGCACTCTACTCT	
MTRNR1 Section 3	GCAAACCCTGATGAAGGCTA	GCGCCAGGTTTCAATTTCTA	
MTTS1	GGCTACACCCTAG ACCAAACC	GGCGTGATCATGAAAGGTG	TD-54: 94 ºC - 5 minutes, 5 cycles of 94ºC-30s, 64 ºC-30s, 72 º-30s, then 30 cycles of 94ºC-30s, 54 ºC-30s, 72 ºC-30s and finally 72ºC for 7 minutes and a soak

(retrieved from http://www.ncbi.nlm.nih.gov/genbank/ on February 16, 2011)

Appendix 1: The Forward and Reverse primers and PCR conditions for each gene.

Gene	Forward Primer	Reverse Primer	PCR Conditions
GJB3 1.1	AGTCTGCCCCTCTGAGTCAC	AAGGCTCCAATGAGATCAGG	TD54, with Betaine, 5%DMSO
GJB3 1.2	GCTGTGGGTACAAGGTCTGC	GAGCTGGGCTCAGCAGTG	TD54, with Betaine, 5%DMSO
GJB3 1.3	CGGCACTTAACAGTTTGCAG	GGGAGGATTGAGAGGTACGG	TD54, with Betaine, 5%DMSO
GJB3 AltUTR	GACAGCACCCAAGGGTAGAA	TTAGTAGCTGGGCGACTTCC	TD54, with Betaine, 5%DMSO
GJB3 2.1	GAACTCAGAACACTGCCTGGT	AGGGGCATGTGACGAAGAT	TD54, with Betaine, 5%DMSO
GJB3 2.2	GGACTTTGACTGCAACACCA	GGCAGATGAGGTAGCAGAGC	TD54, with Betaine, 5%DMSO
GJB3 2.3	TCTGGCATGGCTTCAATATG	TCACTCAGCCCCTGTAGGAC	TD54, with Betaine, 5%DMSO
GJB3 2.4	ACCCAGGCAATAACAAGCTG	ATTCTCAGCCCTCCAGACCT	TD54, with Betaine, 5%DMSO
GJB3 2.5	TCAACCAGGAAGGGATCAAC	CCCTTTCTGAGGGCTTACCT	TD54, with Betaine, 5%DMSO
TMC1 Exon 1	TGTAAAACGACGGCCAGTGGGTGACAGAGCAAGACTCC	CAGGAAACAGCTATGACCTCTCATGGAGAAAGTCTTCGATT	TD54, with Betaine, 5%DMSO
TMC1 Exon 2	TGTAAAACGACGGCCAGTGTCTTCACGCAGCCAGC	CAGGAAACAGCTATGACCGTACCCTTAGGTGGATCACTTG	TD54, with Betaine, 5%DMSO
TMC1 Exon 3	TGTAAAACGACGGCCAGTAAACTGTGACAGGGTCAGGG	CAGGAAACAGCTATGACCGAAAATGCATCACTTACAAATTAGG	TD54, with Betaine, 5%DMSO

Gene	Forward Primer	Reverse Primer	PCR Conditions
TMC1 Exon 4	TGTAAAACGACGGCCAGTACTCCTGTTGCATTCCTTGG	CAGGAAACAGCTATGACCCCCATTCCATACAATCCTGC	TD54, with Betaine, 5%DMSO
TMC1 Exon 5	TGTAAAACGACGGCCAGTTGGTGAAATGGCACCAGG	CAGGAAACAGCTATGACCAAAGGGAAAGCCCCAAAAG	TD54, with Betaine, 5%DMSO
TMC1 Exon 6	TGTAAAACGACGGCCAGTAAATCGTGCTCATTTTGGC	CAGGAAACAGCTATGACCAAAGTTATCCACACACACTTCTGC	TD54, with Betaine, 5%DMSO
TMC1 Exon 7	TGTAAAACGACGGCCAGTATCACGATGTGGAGAATTGC	CAGGAAACAGCTATGACCGCATCATCAGATTAAGGCTCTC	TD54, with Betaine, 5%DMSO
TMC1 Exon 8	TGTAAAACGACGGCCAGTCAGGAAGGCAAACACTACTGC	CAGGAAACAGCTATGACCTGCTTCCAATTTATAATCATTAGCC	TD54, with Betaine, 5%DMSO
TMC1 Exon 9	TGTAAAACGACGGCCAGTAAGAAATAAGACTGCAGACCTGG	CAGGAAACAGCTATGACCTCCCCTGTAAATTTCAATACCAC	TD54, with Betaine, 5%DMSO
TMC1 Exon 10	TGTAAAACGACGGCCAGTGCTGCCAGAGAGACATTTCC	CAGGAAACAGCTATGACCTTCCCAAAAGTCCAGAAACTG	TD54, with Betaine, 5%DMSO
TMC1 Exon 11	TGTAAAACGACGGCCAGTAAAAGGACCAATGCCTCAC	CAGGAAACAGCTATGACCTCCTATGACTCTAAGACGTGAAAATAG	TD54, with Betaine, 5%DMSO
TMC1 Exon 12	TGTAAAACGACGGCCAGTGACCCCACATGGAGACCC	CAGGAAACAGCTATGACCAGACATTCAGCCTGACCCAG	TD54, with Betaine, 5%DMSO
TMC1 Exon 13	TGTAAAACGACGGCCAGTATCAACATGGCAGCTGAAAC	CAGGAAACAGCTATGACCGGCCTGTGTTTATGTTCAGTTC	TD54, with Betaine, 5%DMSO
TMC1 Exon 14	TGTAAAACGACGGCCAGTTATTGCTTCTCCACTTCAACAC	CAGGAAACAGCTATGACCTTGGTAGGCAGAAACCATGAG	TD54, with Betaine, 5%DMSO
TMC1 Exon 15	TGTAAAACGACGGCCAGTCATTGTCATTCCTCACATTCTG	CAGGAAACAGCTATGACCCTGTAAGGGCAGGATAGGGG	TD54, with Betaine, 5%DMSO

Gene	Forward Primer	Reverse Primer	PCR Conditions
TMC1 Exon 16	TGTAAAACGACGGCCAGTGCCTAGCTCAGAATCTTCCAAA	CAGGAAACAGCTATGACCCAAAGGCATTTCTGGCAAAC	TD54, with Betaine, 5%DMSO
TMC1 Exon 17	TGTAAAACGACGGCCAGTCCAGTCTCAAGTTTGCCAGA	CAGGAAACAGCTATGACCTTCAGAGCCAGCACACAGTC	TD54, with Betaine, 5%DMSO
TMC1 Exon 18	TGTAAAACGACGGCCAGTAATTGCAGTCTTCAAGCCAAT	CAGGAAACAGCTATGACCCAAATCCCCCTCTGTGAGAA	TD54, with Betaine, 5%DMSO
TMC1 Exon 19	TGTAAAACGACGGCCAGTCTGCTATTGTTGCTGAAGGG	CAGGAAACAGCTATGACCGACACCGATTGTATTCTCCTCTAAC	TD54, with Betaine, 5%DMSO
TMC1 Exon 20	TGTAAAACGACGGCCAGTTCTGGTTGAAAGTGGCAGTG	CAGGAAACAGCTATGACCGGATCTCATTTCCACCAACC	TD54, with Betaine, 5%DMSO
TMC1 Exon 21	TGTAAAACGACGGCCAGTTTCCTACCCTGAAAGAGGACC	CAGGAAACAGCTATGACCGAACCCTTAGGGAGAGTGAGAG	TD54, with Betaine, 5%DMSO
TMC1 Exon 22-23	TGTAAAACGACGGCCAGTTCATCCCTATGTCATCCTGAA	CAGGAAACAGCTATGACCGTGATGCCTGAGAAGGGCTA	TD54, with Betaine, 5%DMSO

Appendix 2: **NL Hearing Loss Study Medical Questionnaire**

Newfoundland and Labrador

Hearing Loss Study

Medical Information Questionnaire

The information we are asking you to provide in this questionnaire could help to find the cause of the hearing loss in your family. Please don't be discouraged from completing the questionnaire if you do not know the answers to all the questions - just fill in as much as you can. Any information you provide will be beneficial.
We are always available to answer questions and we can complete the questionnaire with you over the phone, if you prefer.

Adapted from:

THE HARVARD CENTRE
FOR HEREDITARY HEARING LOSS

Appendix 2: NL Hearing Loss Study Medical Questionnaire (cont'd)

SECTION 1 - GENERAL INFORMATION

1. Your Name_____ Date of Birth_____

   Address_____

   Home Phone_____ Work Phone_____

   E-mail Address (if you have one) _____

2. To your knowledge, are your parents related, even distantly? ☐ Yes ☐ No ☐ Don't Know

   (This may sound like a strange question, but in a genetic study, we ask it of everyone)

Please answer the following questions as best you can. If you have seen the doctor, please give us the name and address, if possible. If you think you have seen them but you are not sure of their names, or when you saw them, just indicate approximate date, for example, "*saw an audiologist 10 years ago in Grand Falls*". If you have not had an appointment with the medical person listed, tick no and move to the next question. Any information you can provide will be helpful.

3. Have you ever visited any of the following doctors?

			(Don't Know)
- An ENT Doctor? (Ear, Nose and Throat)	☐ Yes	☐ No	☐ D/K
If yes, where did you see them:_____			
- An Audiologist? (Person performing hearing tests)	☐ Yes	☐ No	☐ D/K
If yes, where did you see them:_____			
- An Eye Doctor? (Ophthalmologist)	☐ Yes	☐ No	☐ D/K
If yes, where did you see them:_____			
- A Genetics Doctor? (Geneticist)	☐ Yes	☐ No	☐ D/K
If yes, where did you see them:_____			
- A doctor who treats diseases of the nervous system? (Neurologist)	☐ Yes	☐ No	☐ D/K
If yes, where did you see them:_____			
- A Heart Doctor? (Cardiologist)	☐ Yes	☐ No	☐ D/K
If yes, where did you see them:_____			

4. Have you ever been admitted to hospital? If yes, please give name of hospital and approximate date(s) of admisison.

   _____

   _____

Appendix 2: NL Hearing Loss Study Medical Questionnaire (cont'd)

SECTION II – MEDICAL HISTORY

Please answer as many of the following questions as you can about your hearing loss. If you don't know the answer to the question, write don't know or d/k next to it and go to the next question. Any information you can provide will be helpful.

A. Hearing History - Please **circle** the term that best describes your hearing at the present time:

5. Left Ear: normal less than normal

   Right ear: normal less than normal

If hearing is less than normal, what is used to improve hearing?

6. Left Ear: nothing hearing aid cochlear implant other _____

   Right Ear: nothing hearing aid cochlear implant other _____

7. Were you born with hearing loss?..... ☐ Yes ☐ No

   If yes, tick affected ear..... ☐ Right ☐ Left

   If no, when did it start?..... ☐ During Childhood ☐ During Teen Years ☐ During Adulthood

8. Did your hearing loss begin during or soon after:

			(Not Applicable)
- being pregnant.....	☐ Yes	☐ No	☐ N/A
- an airplane flight.....	☐ Yes	☐ No	☐ N/A
- scuba diving.....	☐ Yes	☐ No	☐ N/A
- intravenous antibiotic treatment.....	☐ Yes	☐ No	☐ N/A
- chemotherapy for cancer.....	☐ Yes	☐ No	☐ N/A
- a severe infection, such as meningitis?.....	☐ Yes	☐ No	☐ N/A
- exposure to a **sudden** loud noise.....	☐ Yes	☐ No	☐ N/A
- **prolonged** exposure to loud noise.....	☐ Yes	☐ No	☐ N/A
- an ear infection.....	☐ Yes	☐ No	☐ N/A
- ear surgery (including insertion of T-tubes)...	☐ Yes	☐ No	☐ N/A
- injury to the head or the ear.....	☐ Yes	☐ No	☐ N/A

Appendix 2: NL Hearing Loss Study Medical Questionnaire (cont'd)

Pattern of Hearing Loss. Please tell us which ear has hearing loss by answering the following questions.

9. Your hearing is:

			(Not Applicable)
- Stable (has not changed much over several years). ...	☐ Right	☐ Left	N/A
- Fluctuating (sometimes better, sometimes worse).....	☐ Right	☐ Left	N/A
- Slowly progressing (getting worse over years).....	☐ Right	☐ Left	N/A
- Rapidly progressing (getting worse over weeks/months).....	☐ Right	☐ Left	N/A
- Sudden hearing loss	☐ Right	☐ Left	N/A

B. Patient's Medical History. Have you ever had any of the following:

10.

			(Don't Know)
- Scarlet fever.....	☐ Yes	☐ No	☐ D/K
- Measles or German measles (circle which one).....	☐ Yes	☐ No	☐ D/K
- Mumps.....	☐ Yes	☐ No	☐ D/K
- Meningitis (brain infection).....	☐ Yes	☐ No	☐ D/K
- Tuberculosis (TB).....	☐ Yes	☐ No	☐ D/K
- Repeated ear infections.....	☐ Yes	☐ No	☐ D/K
- Premature graying of hair before age 30..... (Not just at the temples)	☐ Yes	☐ No	☐ D/K
- Kidney problems.....	☐ Yes	☐ No	☐ D/K
- Diabetes mellitus ("sugar diabetes").....	☐ Yes	☐ No	☐ D/K
- Thyroid problems (goiter, under active, overactive)....	☐ Yes	☐ No	☐ D/K
- Depression or "nerves".....	☐ Yes	☐ No	☐ D/K

11. Is there anything else which you think we should know about your medical history?

Appendix 2: NL Hearing Loss Study Medical Questionnaire (cont'd)

Faculty of Medicine, Schools of Nursing and Pharmacy of Memorial University of Newfoundland; Health Care Corporation, St. John's; Newfoundland Cancer Treatment and Research Foundation

Consent to Take Part in Health Research

TITLE: The Genetics of Hereditary Deafness in Newfoundland

INVESTIGATOR(S):

SPONSOR:

You have been asked to take part in a research study. It is up to you to decide whether to be in the study or not. Before you decide, you need to understand what the study is for, what risks you might take and what benefits you might receive. This consent form explains the study.

The researchers will:

- **discuss the study with you**
- **answer your questions**
- **keep confidential any information which could identify you personally**
- **be available during the study to deal with problems and answer questions**

If you decide not to take part or to leave the study this will not affect your health care.

1. Introduction/Background:

Some forms of deafness are hereditary. That means that they are inherited in families as a result of an altered gene. A gene is a piece of genetic material (DNA) which is passed from parents to children. If we can identify the genes that are altered in each family, it would allow us to better understand the process of hearing. We might also learn what goes wrong in some forms of deafness and how it might be treated.

2. Purpose of study:

Our goal is to identify the genes involved in hereditary deafness in families.

3. Description of the study procedures and tests:

If you agree to take part in this study, you will be asked to:

- Tell us about your hearing and the hearing of other members of your family, and other related aspects of your health.
- Have your hearing tested by a registered audiologist.
- Have a blood sample drawn for DNA testing.
- Complete a hearing loss questionnaire.

We might also want to review your medical records related to your deafness.

Initials: _____

Appendix 2: NL Hearing Loss Study Medical Questionnaire (cont'd)

4. **Length of time:**
   If you take part in this study, the interview will last 30-60 minutes including the blood sampling. The hearing test will take about 30 minutes. It will be arranged at a time that is convenient for you. The research may take us several years but you will not have to be involved again. We will keep you informed of our findings.

5. **Possible risks and discomforts:**
   The only discomfort is that of giving a blood sample.

6. **Benefits:**
   It is not known whether this study will benefit you.

7. **Liability statement:**
   Signing this form gives us your consent to be in this study. It tells us that you understand the information about the research study. When you sign this form, you do not give up your legal rights. Researchers or agencies involved in this research study still have their legal and professional responsibilities.

8. **Compensation:**
   In the event that you suffer injury as a direct result of taking part in this study, necessary medical treatment not covered by provincial health care insurance will be available at no additional cost to you.

9. **Confidentiality:**
   Unless required by law, only the researchers may have access to any confidential documents pertaining to your participation in this study that may identify you by name. Furthermore, your name will not appear in any report or article published as a result of this study.

10. **Genetic Studies:**
    In order to interpret the results of genetic research, we need to have correct information about parents. Sometimes the research shows new information about birth parents. This could happen in the case of an adoption or a mistake in the identity of a mother or father. This information will not be given to anyone including you or other family members.

11. **Future use of tissue/DNA samples.**

In order to preserve a valuable resource, your (tissue/DNA) samples may be stored at the end of this research project. It is possible that these samples may be useful in a future research project dealing with hereditary deafness which may or may not be related to the current research project. **Any future research would have to be approved by a Research Ethics Board (REB).**

Please tick **one** of the following options:

	I agree that my (tissue/DNA) samples can be used for any approved research project but only if I am contacted again to give consent for the new project.
	I agree that my (tissue/DNA) sample can be used for any approved research project without contacting me again, but **only if my name* cannot be linked, in any way, to the sample.**
	Under no circumstances may my sample by used for future research. **My sample must be destroyed** at the end of this present project.

*Includes name, MCP number or any other identifying information.

The DNA sample from this study will be stored in St. John's, Newfoundland and Seattle, Washington for an indefinite period of time.

11. Contact Information:

If you have any questions about taking part in this study, you can meet with the investigator who is in charge of the study at this institution. That person is:

Or you can talk to someone who is not involved with the study at all, but can advise you on your rights as a participant in a research study. This person can be reached through:

Initials: _____

Appendix 2: NL Hearing Loss Study Medical Questionnaire (cont'd)

Signature Page

Study title: **The Genetics of Hereditary Deafness in Newfoundland**

Name of principal investigator: **Dr. Terry-Lynn Young**

To be filled out and signed by the participant:

	Please check as appropriate:
I have read the consent [and information sheet].	Yes { } No { }
I have had the opportunity to ask questions/to discuss this study.	Yes { } No { }
I have received satisfactory answers to all of my questions.	Yes { } No { }
I have received enough information about the study.	Yes { } No { }
I have spoken to Dr. Young or her research assistant and she has answered my questions.	Yes { } No { }
I understand that I am free to withdraw from the study • at any time • without having to give a reason • without affecting my future care	Yes { } No { }
I understand that it is my choice to be in the study and that I may not benefit.	Yes { } No { }
I agree that the study doctor or investigator may read the parts of my hospital records which are relevant to the study.	Yes { } No { }
I agree to take part in this study.	Yes { } No { }

_____ _____

Signature of participant Date

_____ _____

Signature of witness Date

To be signed by the investigator:

I have explained this study to the best of my ability. I invited questions and gave answers. I believe that the participant fully understands what is involved in being in the study, any potential risks of the study and that he or she has freely chosen to be in the study.

_____ _____

Signature of investigator Date

Telephone number: _____

Assent of minor participant (if appropriate):

_____ _____

Signature of minor participant Date

_____ _____

Relationship to participant named above Age

Initials: _____

Appendix 3: Standard DNA Extraction Protocol

Purpose: To provide a method for the extraction, precipitation, and re-suspension of DNA from (EDTA) whole blood.

Responsibilities: Lab personnel who have been trained in this procedure.

Definitions:

- hr. = hour
- ppt = precipitate
- WBC = white blood cell

Equipment and Supplies:

Equipment:

- centrifuge
- Biological safety cabinet
- 37º or 55 ^{oC} water bath or heat block
- Pipette aid

Supplies:

- 50 ml &15ml centrifuge tubes
- 1 & 10ml pipettes
- 2 ml micrewtube with o-ring seal screw cap
- "hooked" Pasteur pipettes
- 500ml plastic beaker

Reagents:

- RBC lysis solution (NH_4CL/Tris)
- 0.85% NaCl
- Nuclei lysis buffer (10mM Tris-HCl, 400mM NaCl, 2.0 mM EDTA, pH 8.0).
- 10%SDS
- Protease solution (3mg/ml protease, 1% SDS, 2mM EDTA)
- Saturated NaCl
- Absolute ethanol
- TE buffer
- 70% ethanol

- TE buffer (10mM Tris, 1mM EDTA, pH 8.0)
- Bleach (for disinfection).

Procedure:

Note: *place a 1.0 litre plastic beaker containing ~100ml of bleach in the safety cabinet for waste discard/disinfection. Decant all supernatants etc. into this container.*

1. Pre-warm RBC lysis solution to 37ºC.
2. In a 50ml centrifuge tube mix 5 vols. of RBC lysis solution with 1 vol. of whole blood (to avoid aerosols add the lysis solution first then the blood). Mix by inverting and then incubate at 37ºC for 5min to 1 hr.
3. Centrifuge at 1000 x g (2500 rpm) for 5min.
4. Decant the supernatant and add 10 ml of 0.85% NaCl. Vortex (vigorously) to re-suspend the WBC pellet then centrifuge (as previous).
5. Decant the supernatant and add 3ml of nuclei lysis buffer
6. Vortex (vigorously) to re-suspend the WBC pellet then and add 0.2 ml of 10% SDS and 0.5ml of Protease solution.
7. Incubate at 55ºC for 2 hr. or 37ºC for 12 to 60 hr.
8. Add 1.0ml of saturated NaCl, shake/vortex vigorously for ~15sec. then centrifuge at 1000 x g (~2500 rpm) for 15min's.
9. Carefully (so as not to disturb the pellet) decant the supernatant into a clean 15ml centrifuge tube containing 2 volumes of absolute ethanol and gently mix by inversion until a DNA ppt. is visible. If the ppt. is not visible check the underside of the tube cap – sometimes the DNA lodges there!
10. "Hook" the DNA ppt. with a ("hooked") Pasteur pipette, decant the waste from the tube, and then place the inverted pipette back into the tube.
11. Wash the DNA with a stream of (~ 2-5ml) of 70% ethanol (the ethanol will flow down the pipette and into the tube).
12. Break off the hooked end (containing the DNA) of the pipette into a 2 ml micrewtube with o-ring seal screw cap. Add 1.0ml of TE buffer and store at 4ºC.
13. DNA can be quantitated (UV 260/280nm) after 24 hr storage at 4ºC or >2hr. at 37ºC.

Potential Problems and Corrective Action:

Potential Problem: DNA remains as a viscous "glob" in tube.

Corrective Action: Incubate at 56 ºC for 1 hr. Or overnight at 37 ºC.

Potential Problem: DNA remains as a viscous "glob" in tube even after incubation at 56 ºC for 1 hr. Or overnight at 37 ºC.

Corrective Action: Add T.E. in 250µl increments; followed by incubation at 56 ºC for 1hr. until DNA dissolves.

Appendix 4: **Full Pedigrees for HL Families**

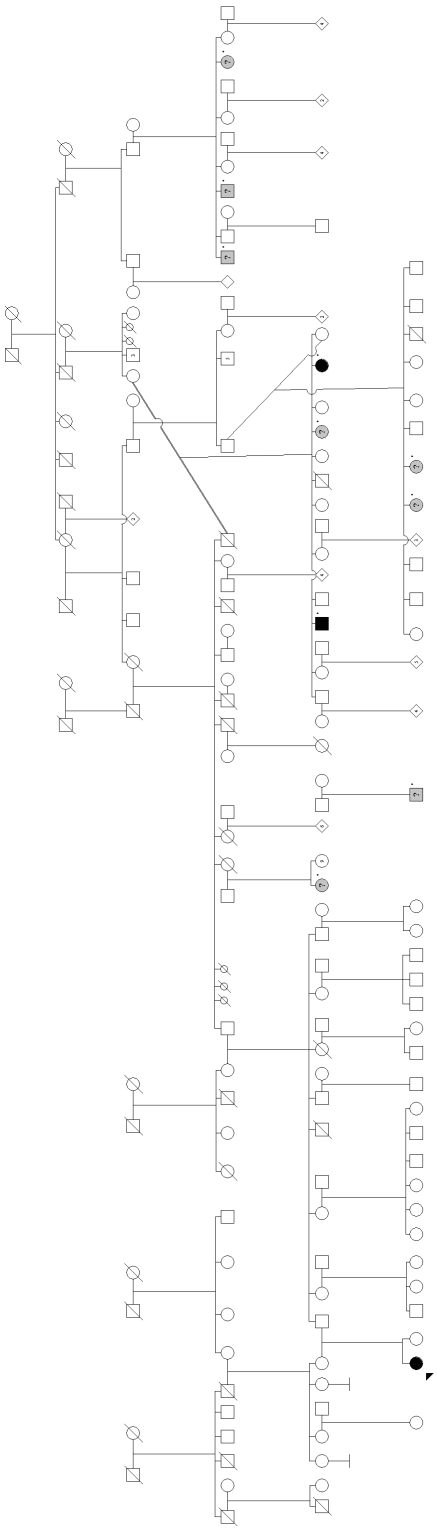

Figure 1. The full pedigree for family 2155. The proband in this family has the 35delG mutation in connexin 26 as well as (del13S1830) in connexin 30

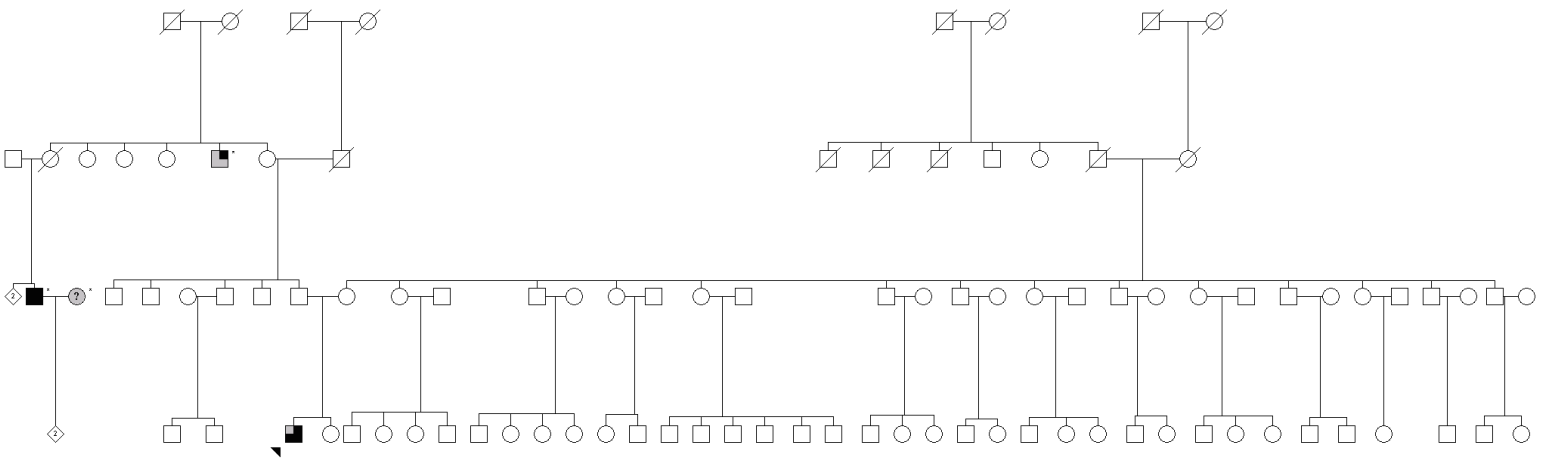

Figure 2. The full pedigree for family 2167. The proband in this family has a G951A mutation in *MTRNR1*.

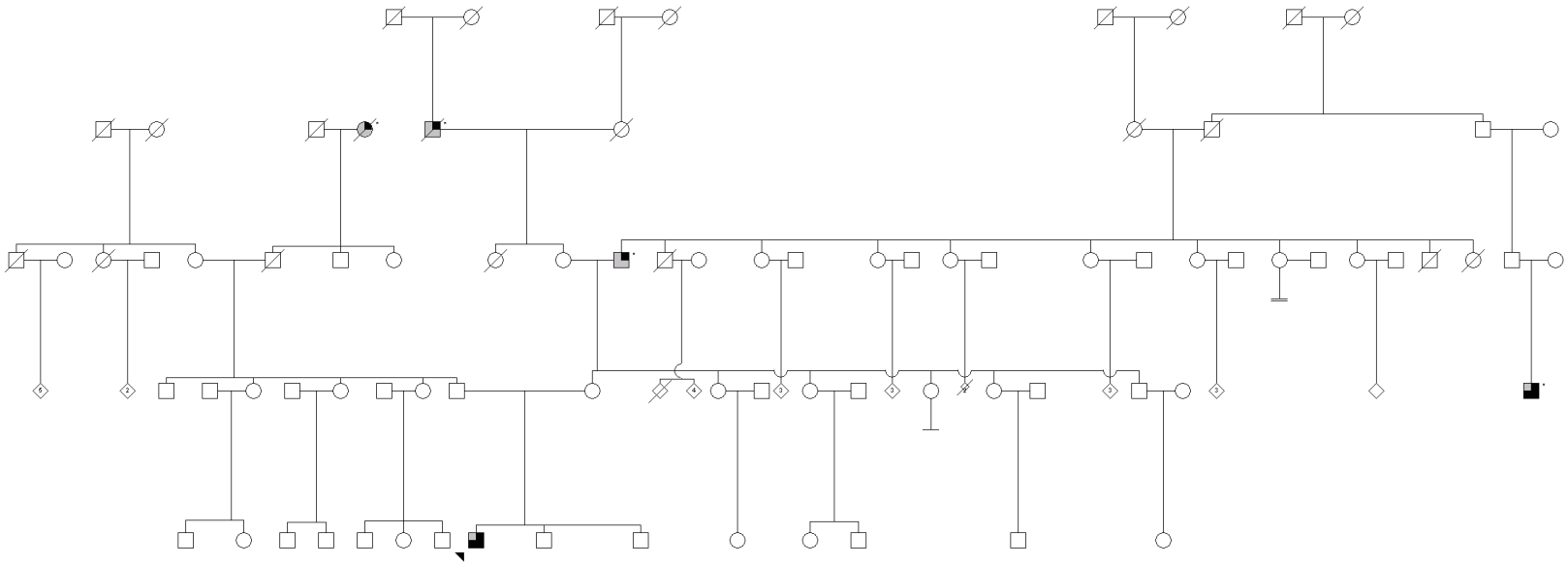

Figure 3. The full pedigree for family 2097. The proband in this family has the R32W mutation in connexin 31

Appendix 5: **DNA sample numbers and their corresponding Family numbers for all probands screened along with what mutations were found (both previous and in this study)**

Genes previously found to cause hearing loss in NL Population

		WFS1	*TMPRSS3*		*PCDH15*
DNA	**Family**	**p. 2146 G→A**	**207delC**	**IVS8 + 8insT**	**V528D**
NWE-01	2047	X	X	X	X
NWF-01	2052	X	X	X	X
03MG600	2045	X	X	X	X
00MG1626	2049	X	X	X	X
03MG958	2065	X	X	X	X
03MG1424	2055	X	X	X	X
02MG802	2058	X	X	X	X
03MG1940	2046	X	X	X	X
02MG2269	2057	X	X	X	X
04MG207	2062	X	X	X	X
RR04-35	2060	X	X	X	X
CT04-37	2072	X	X	X	X
ME04-40	2075	X	X	X	X
JT04-49	2010	X	X	X	X
JM04-62	2064	X	X	X	X
MK04-73	2054	X	X	X	X
RN04-78	2069	X	X	X	X
NT04-84	2071	X	X	X	X
LH04-83	2029	X	X	X	X
TR04-88	2076	X	X	X	X
BM04-169	2078	X	X	X	X
SO04-172	2067	X	X	X	X
JT04-186	2077	X	X	X	X
WB04-199	2079	X	X	X	X
TO04-197	2080	X	X	X	X
AM04-258	2070	X	X	X	X
VB04-268	2082	X	X	X	X
TS05-5	2083	X	X	X	X
BC05-33	2024	X	X	X	X
04MG1207	2084	X	X	X	X
04MG2005	2033	X	X	X	X
04MG1911	2089	X	X	X	X
04MG2030	2090	X	X	X	X
00MG440	2066	X	X	X	X
04MG2043	2091	X	X	X	X
RB04-100	2041	X	X	X	X
AN06-18	2068	X	X	X	X
01MG1188	2053	X	X	X	X
02MG109	2074	X	X	X	X
IT06-28	2048	X	X	X	X
CP06-93	2092	X	X	X	X
RK06-113	2093	X	X	X	X
JS06-138	2095	X	X	X	X
BR06-148	2096	X	X	X	X
LR06-153	2073	X	X	X	X
KM06-227	2094	X	X	X	X
SR07-13	2097	X	X	X	X
KG07-77	2100	X	X	X	X
DB07-174	2102	X	X	X	X
AB07-180	2099	X	X	X	X

Genes previously found to cause hearing loss in the NL population cont'd

		WFS1 p. 2146 G→A	*TMPRSS3* 207delC	IVS8 + 8insT	*PCDH15* V528D
MG08-09	2109	X	X	X	X
MN08-44	2104	X	X	X	X
MB08-66	2110	X	X	X	X
TP08-80	2081	X	X	X	X
RH08-134	2001	X	X	X	X
DB08-156	2103	X	X	X	X
DB08-197	2105	X	X	X	X
WC08-13	2111	X	X	X	X
DJ08-40	2117	X	X	X	X
DJ08-51	2120	X	X	X	X
TC08-55	2124	X	X	X	X
RC08-61	2088	X	X	X	X
KB08-65	2126	X	X	X	X
SC08-68	2127	X	X	X	X
MB08-81	2112	X	X	X	X
RP08-92	2129	X	X	X	X
SS08-123	2135	X	X	X	X
OH08-124	2144	X	X	X	X
BB08-126	2143	X	X	X	X
BM08-127	2146	X	X	X	X
WT08-132	2140	X	X	X	X
JH08-137	2142	X	X	X	X
JD08-139	2113	X	X	X	X
PK08-142	2133	X	X	X	X
KM08-144	2149	X	X	X	X
CS08-149	2161	X	X	X	X
DR08-152	2156	X	X	X	X
WT08-153	2160	X	X	X	X
EM08-154	2114	X	X	X	X
GB08-155	2152	X	X	X	X
GH08-157	2131	X	X	X	X
DB08-193	2151	X	X	X	X
KS08-195	2171	X	X	X	X
DA08-201	2167	X	X	X	X
RH08-210	2173	X	X	X	X
RP08-212	2155	X	X	X	X
FC08-213	2169	X	X	X	X
WM08-215	2164	X	X	X	X
JO08-216	2177	X	X	X	X
JH08-220	2182	X	X	X	X
AC08-223	2180	X	X	X	X
JK08-229	2183	X	X	X	X
03MG365	2178	X	X	X	X
GH08-233	2189	X	X	X	X
MA08-234	2145	X	X	X	X
MR08-235	2136	X	X	X	X
EF08-238	2141	X	X	X	X
MT08-239	2134	X	X	X	X
08MG1184	2176	X	X	X	X
BM09-25	2179	X	X	X	X

Genes previously found to cause hearing loss in the NL population cont'd

		WFS1 **p. 2146 G→A**	***TMPRSS3*** **207delC**	**IVS8 + 8insT**	***PCDH15*** **V528D**
CR09-48	2158	X	X	X	X
TA09-46	2194	X	X	X	X
EW09-43	2197	X	X	X	X
JR09-45	2196	X	X	X	X
09MG1707	2207	X	X	X	X
00MG1300	2157	X	X	X	X
720	2162	X	X	X	X
BY10-42	2185	X	X	X	X
09MG1785	2210	X	X	X	X
DG09-133	2206	X	X	X	X

Mutations in Connexin 26 and Connexin 30

		Cx26		*Cx30*
DNA	**Family**	**Ex.2:35delG**	**Ex. 2 NP 249 C>G: F83L**	**delD1351830**
NWE-01	2047	X	X	X
NWF-01	2052	X	X	X
03MG600	2045	X	X	X
00MG1626	2049	X	X	X
03MG958	2065	X	X	X
03MG1424	2055*	X het.	X	X het.
02MG802	2058*	X hom.	X	X
03MG1940	2046	X	X	X
02MG2269	2057	X	X	X
04MG207	2062	X	X	X
RR04-35	2060	X	X	X
CT04-37	2072	X	X	X
ME04-40	2075	X	X	X
JT04-49	2010	X	X	X
JM04-62	2064	X	X	X
MK04-73	2054	X	X	X
RN04-78	2069	X	X	X
NT04-84	2071	X	X	X
LH04-83	2029	X	X	X
TR04-88	2076	X	X	X
BM04-169	2078	X	X	X
SO04-172	2067*	X het.	X	X het.
JT04-186	2077	X	X	X
WB04-199	2079	X	X	X
TO04-197	2080	X	X	X
AM04-258	2070	X	X	X
VB04-268	2082	X	X	X
TS05-5	2083	X	X	X
BC05-33	2024	X	X	X
04MG1207	2084	X	X	X
04MG2005	2033	X	X	X
04MG1911	2089	X	X	X
04MG2030	2090	X	X	X
00MG440	2066	X	X	X
04MG2043	2091	X	X het.	X
RB04-100	2041	X	X	X
AN06-18	2068*	X het.	X	X het.
01MG1188	2053	X	X	X
02MG109	2074*	X het.	X	X
IT06-28	2048	X	X	X
CP06-93	2092*	X het.	X	X
RK06-113	2093	X	X	X
JS06-138	2095*	X hom.	X	X
BR06-148	2096	X	X	X
LR06-153	2073	X	X	X
KM06-227	2094	X	X	X
SR07-13	2097*	X	X	X het.

*Found in screening previous to study

Mutations in Connexin 26 and Connexin 30 cont'd

		Cx26 Ex.2:35delG	Ex. 2 NP 249 C>G: F83L	*Cx30* delD1351830
KG07-77	2100	X	X	X
DB07-174	2102	X	X	X
AB07-180	2099	X	X	X
MG08-09	2109	X	X	X
MN08-44	2104	X	X	X
MB08-66	2110	X	X	X
TP08-80	2081	X	X	X
RH08-134	2001	X	X	X
DB08-156	2103	X	X	X
DB08-197	2105	X	X	X
WC08-13	2111	X	X	X
DJ08-40	2117	X	X	X
DJ08-51	2120	X	X	X
TC08-55	2124	X	X	X
RC08-61	2088	X	X	X
KB08-65	2126	X	X	X
SC08-68	2127	X	X	X
MB08-81	2112	X	X	X
RP08-92	2129	X	X	X
SS08-123	2135	X	X	X
OH08-124	2144	X	X	X
BB08-126	2143	X	X	X
BM08-127	2146	X	X	X
WT08-132	2140	X	X	X
JH08-137	2142	X	X	X
JD08-139	2113	X	X	X
PK08-142	2133	X	X	X
KM08-144	2149	X	X	X
CS08-149	2161	X	X	X
DR08-152	2156	X	X	X
WT08-153	2160	X	X	X
EM08-154	2114	X	X	X
GB08-155	2152	X	X	X
GH08-157	2131	X	X	X
DB08-193	2151	X	X	X
KS08-195	2171	X	X	X
DA08-201	2167	X	X	X
RH08-210	2173	X	X	X
RP08-212	2155	X het.	X	X het.
FC08-213	2169	X	X	X
WM08-215	2164	X	X	X
JO08-216	2177	X	X	X
JH08-220	2182	X	X	X
AC08-223	2180	X	X	X
JK08-229	2183	X	X	X
03MG365	2178	X	X	X
GH08-233	2189	X	X	X

Mutations in Connexin 26 and Connexin 30 cont'd

		Cx26 Ex.2:35delG	Ex. 2 NP 249 C>G: F83L	*Cx30* delD1351830
MA08-234	2145	X	X	X
MR08-235	2136	X	X	X
EF08-238	2141	X	X	X
MT08-239	2134	X	X	X
08MG1184	2176	X	X	X
BM09-25	2179	X	X	X
CR09-48	2158	X	X	X
TA09-46	2194	X	X	X
EW09-43	2197	X het.	X	X
JR09-45	2196	X	X	X
09MG1707	2207	X	X	X
00MG1300	2157	X	X	X
720	2162	X	X	X
BY10-42	2185	X	X	X
09MG1785	2210	X	X	X
DG09-133	2206	X	X	X

Mutations in the Mitochondrial genome

		MTRNR1	*MTTS1*	*Mito D-Loop*
DNA	**Family**	**A1555G**		**C150T & T152C**
NWE-01	2047	X	X	X
NWF-01	2052	X	X	X
03MG600	2045	X	X	X
00MG1626	2049	X	X	X
03MG958	2065	X	X	X
03MG1424	2055	X	X	X
02MG802	2058	X	X	X
03MG1940	2046	X	X	X
02MG2269	2057	X	X	X
04MG207	2062	X	X	X
RR04-35	2060	X	X	X
CT04-37	2072	X	X	**X hom.**
ME04-40	2075	X	X	X
JT04-49	2010	X	X	**X hom.**
JM04-62	2064	X	X	X
MK04-73	2054	X	X	X
RN04-78	2069	X	X	X
NT04-84	2071	X	X	X
LH04-83	2029	X	X	X
TR04-88	2076	X	X	X
BM04-169	2078	X	X	X
SO04-172	2067	X	X	X
JT04-186	2077	X	X	X
WB04-199	2079	X	X	X
TO04-197	2080	X	X	X
AM04-258	2070	X	X	X
VB04-268	2082	X	X	X
TS05-5	2083	X	X	X
BC05-33	2024	X	X	X
04MG1207	2084	X	X	X
04MG2005	2033	X	X	X
04MG1911	2089	X	X	X
04MG2030	2090	X	X	X
00MG440	2066	X	X	X
04MG2043	2091	X	X	X
RB04-100	2041	X	X	X
AN06-18	2068	X	X	X
01MG1188	2053	X	X	X
02MG109	2074	X	X	X
IT06-28	2048	X	X	X
CP06-93	2092	X	X	X
RK06-113	2093	X	X	X
JS06-138	2095	X	X	X
BR06-148	2096	X	X	X
LR06-153	2073	X	X	X
KM06-227	2094	X	X	X
SR07-13	2097	X	X	X
KG07-77	2100	X	X	X
DB07-174	2102	X	X	X
AB07-180	2099	X	X	X

Mutations in the Mitochondrial Genome cont'd

		MTRNR1 A1555G	*MTTS1*	Mito D Loop C150T & T152C
MG08-09	2109	X	X	X
MN08-44	2104	X	X	X
MB08-66	2110	X	X	X
TP08-80	2081	X	X	X
RH08-134	2001	X	X	X
DB08-156	2103	X	X	X
DB08-197	2105	X	X	X
WC08-13	2111	X	X	X
DJ08-40	2117	X	X	X
DJ08-51	2120	X	X	X
TC08-55	2124	X	X	X
RC08-61	2088	X	X	X
KB08-65	2126	X	X	X
SC08-68	2127	X	X	X
MB08-81	2112	X hom.	X	X
RP08-92	2129	X	X	X
SS08-123	2135	X	X	X
OH08-124	2144	X hom.	X	X
BB08-126	2143	X	X	X
BM08-127	2146	X	X	X
WT08-132	2140	X	X	X
JH08-137	2142	X	X	X
JD08-139	2113	X	X	X
PK08-142	2133	X	X	X
KM08-144	2149	X	X	X
CS08-149	2161	X	X	X
DR08-152	2156	X	X	X
WT08-153	2160	X	X	X
EM08-154	2114	X	X	X
GB08-155	2152	X	X	X
GH08-157	2131	X	X	X
DB08-193	2151	X	X	X
KS08-195	2171	X	X	X
DA08-201	2167	X	X	X
RH08-210	2173	X	X	X
RP08-212	2155	X	X	X
FC08-213	2169	X	X	X
WM08-215	2164	X	X	X
JO08-216	2177	X	X	X
JH08-220	2182	X	X	X
AC08-223	2180	X	X	X
JK08-229	2183	X	X	X
03MG365	2178	X	X	X
GH08-233	2189	X	X	X
MA08-234	2145	X	X	X
MR08-235	2136	X	X	X
EF08-238	2141	X	X	X
MT08-239	2134	X	X	X
08MG1184	2176	X	X	X
BM09-25	2179	X	X	X

Mutations in the Mitochondrial Genome cont'd

		MTRNR1 A1555G	*MTTS1*	Mito D Loop C150T & T152C
CR09-48	2158	X	X	X
TA09-46	2194	X	X	X
EW09-43	2197	X	X	X
JR09-45	2196	X	X	X
09MG1707	2207	X	X	X
00MG1300	2157	X	X	X
720	2162	X	X	X
BY10-42	2185	X	X	X
09MG1785	2210	X	X	X
DG09-133	2206	X	X	X

Mutation in Connexin 31 and *TMCI*

		Cx31		*TMC1*		
DNA	**Family**	**V37M**	**R32W**	**R141W**	**G182D**	**c.1763+3 A>G**
NWE-01	2047	X	X	X	X	X
NWF-01	2052	X	X	X	X	X
03MG600	2045	X	X	X	X	X
00MG1626	2049	X	X	X	X	X
03MG958	2065	X	X	X	X	X Het
03MG1424	2055	X	X	X	X	X
02MG802	2058	X	X	X	X	X
03MG1940	2046	X	X	X	X	X
02MG2269	2057	X	X	X	X	X
04MG207	2062	X	X	X	X	X
RR04-35	2060	X	X	X	X	X
CT04-37	2072	X	X	X	X	X
ME04-40	2075	X	X Het	X	X	X
JT04-49	2010	X	X	X Het	X	X
JM04-62	2064	X	X	X	X	X
MK04-73	2054	X	X	X	X	X
RN04-78	2069	X	X	X	X	X
NT04-84	2071	X	X	X	X	X
LH04-83	2029	X	X	X	X	X
TR04-88	2076	X	X	X	X	X
BM04-169	2078	X	X Het	X	X	X
SO04-172	2067	X	X	X	X	X
JT04-186	2077	X	X	X	X	X
WB04-199	2079	X	X	X	X	X
TO04-197	2080	X	X	X	X	X
AM04-258	2070	X	X	X	X	X
VB04-268	2082	X	X	X	X	X
TS05-5	2083	X Het.	X	X	X	X
BC05-33	2024	X	X	X	X	X
04MG1207	2084	X	X	X	X	X
04MG2005	2033	X	X	X	X	X
04MG1911	2089	X	X	X	X	X
04MG2030	2090	X	X	X	X	X
00MG440	2066	X	X	X	X	X
04MG2043	2091	X	X	X	X	X
RB04-100	2041	X	X	X	X	X
AN06-18	2068	X	X	X	X	X
01MG1188	2053	X	X	X	X	X
02MG109	2074	X	X	X	X	X
IT06-28	2048	X	X	X	X	X
CP06-93	2092	X	X	X Het	X	X
RK06-113	2093	X	X	X	X	X
JS06-138	2095	X	X	X	X	X
BR06-148	2096	X	X	X	X	X
LR06-153	2073	X	X	X	X	X
KM06-227	2094	X	X	X	X	X
SR07-13	2097	X	X Het	X	X	X
KG07-77	2100	X	X	X	X	X
DB07-174	2102	X	X	X	X	X
AB07-180	2099	X	X	X	X	X

Mutation in Connexin 31 and *TMCI* cont'd

		Cx31 V37M	R32W	*TMC1* R141W	G182D	c.1763+3 A>G
MG08-09	2109	X	X	X	X	X
MN08-44	2104	X	X	X	X	X
MB08-66	2110	X	X	X	X	X
TP08-80	2081	X	X	X	X	X
RH08-134	2001	X	X	X	X	X
DB08-156	2103	X	X	X	X	X
DB08-197	2105	X	X	X	X	X
WC08-13	2111	X	X	X	X	X
DJ08-40	2117	X	X	X	X	X
DJ08-51	2120	X	X	X	X	X
TC08-55	2124	X	X	X	X Het	X
RC08-61	2088	X	X	X	X	X
KB08-65	2126	X	X	X	X	X
SC08-68	2127	X	X	X	X	X
MB08-81	2112	X	X	X	X	X
RP08-92	2129	X	X	X	X	X
SS08-123	2135	X	X	X	X	X
OH08-124	2144	X	X	X	X	X
BB08-126	2143	X	X	X	X	X
BM08-127	2146	X	X	X Het	X	X
WT08-132	2140	X	X	X	X	X
JH08-137	2142	X	X	X	X	X
JD08-139	2113	X	X	X	X	X
PK08-142	2133	X	X	X	X	X
KM08-144	2149	X	X	X	X	X
CS08-149	2161	X	X	X	X	X
DR08-152	2156	X	X	X	X	X
WT08-153	2160	X	X	X	X	X
EM08-154	2114	X	X	X	X	X
GB08-155	2152	X	X	X	X	X
GH08-157	2131	X	X	X	X	X
DB08-193	2151	X	X	X	X	X
KS08-195	2171	X	X	X	X	X
DA08-201	2167	X	X	X	X	X
RH08-210	2173	X	X	X	X	X
RP08-212	2155	X	X	X	X	X
FC08-213	2169	X	X	X	X	X
WM08-215	2164	X	X	X	X	X
JO08-216	2177	X	X	X Het	X	X
JH08-220	2182	X	X	X	X	X
AC08-223	2180	X	X	X	X	X
JK08-229	2183	X	X	X	X	X
03MG365	2178	X	X	X	X	X
GH08-233	2189	X	X	X	X	X
MA08-234	2145	X	X	X	X	X
MR08-235	2136	X	X	X	X	X
EF08-238	2141	X	X	X	X	X
MT08-239	2134	X	X	X	X	X
08MG1184	2176	X	X	X	X	X
BM09-25	2179	X	X	X	X	X

Mutation in Connexin 31 and *TMCI* cont'd

		Cx31 V37M	R32W	*TMC1* R141W	G182D	c.1763+3 A>G
CR09-48	2158	X	X	X	X	X
TA09-46	2194	X	X	X	X	X
EW09-43	2197	X	X	X	X	X
JR09-45	2196	X	X	X	X	X
09MG1707	2207	X	X	X	X	X
00MG1300	2157	X	X	X	X	X
720	2162	X	X	X	X	X
BY10-42	2185	X	X	X	X	X
09MG1785	2210	X	X	X	X	X
DG09-133	2206	X	X	X	X	X