"INVESTIGATION OF THE GENETIC CAUSE OF HEREDITARY HEARING LOSS IN THREE LARGE DEAF, CONSANGUINEOUS NEWFOUNDLAND FAMILIES"

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Submitted in partial requirement for:

Masters of Science (Medicine)

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January 24, 2008

Abstract

Three large Newfoundland families segregating with autosomal recessive hearing loss were studied in this thesis project: Family A, Family B and Family 41. Previous work on Family A identified a pathogenic mutation in the deafness gene *PCDH15* which explained deafness in five family members homozygous for the mutation but did not fully explain the deafness in five other family members heterozygous for the mutation. A second deafness gene, *CDH23* is located very close to the *PCDH15* on chromosome 10. Single mutations in these two genes are known to cause both Ushers syndrome and non-syndromic deafness. All 69 exons and all intron/exons boundaries in *CDH23* were sequenced in four Family A members which identified 45 SNPs. Only eight SNPs were potentially pathogenic because they were found in the coding region and they were polymorphic. However, no one variant of the eight SNPs segregated exclusively with deafness; in addition, all eight SNPs were previously reported as non-pathogenic. It was concluded that *CDH23* does not contribute to the deafness in Family A. Previous work on Family B determined the familial deafness was due to mutations in *TMPRSS3:* c.782+3delGAG, a novel mutation, and c.207delC, a known mutation. Informative markers and intrageneic SNPs with the *TMPRSS3* gene were used to construct and characterize the two *TMPRSS3* mutation haplotypes. A single copy of the novel *TMPRSS3* mutation (c.782+3delGAG) was found in a deaf boy in Family 41 and his hearing mother and their *TMPRSS3* haplotypes were constructed. It was found that carriers of the *TMPRSS3* c.782+3delGAG mutation in Family B and Family 41 shared a haplotype spanning 10.1Mb. Since the two families are not known to be related, the *TMPRSS3* c.782+3delGAG was designated a founder mutation.

Acknowledgments

This graduate program was an enjoyable experience, with many unanticipated rewards. I would like to take this opportunity to thank the members of the Young lab, who were instrumental in the completion of this project. Firstly, I would like to thank the lab staff, including Mr. Dante Galutira, Dr. Jane Gamberg and Ms. Ingrid Pardoe for their helpful instruction and never-wavering encouragement. Secondly, I would like to thank the students in the lab, including Nancy Merner, Vanessa French and Lance Doucette for their ever-smiling faces and optimistic attitudes. Finally, I would like to thank my supervisor, Dr. Terry-Lynn Young for her open-door policy, exceptional teaching skills and good humor. I will always treasure my experience in "The Young Lab".

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List of Abbreviations and Symbols

ABI – Applied Biosystems International

ARNSHL- Autosomal Recessive Non-Syndromic Hearing Loss

CDH23 – Cadherin23

cM- CentiMorgan

CP- Cytoplasmic

dB-Decibels

dH_20- De-ionized water

dNTPs- Dideoxynucleotide Triphosphates

DNA – Deoxyribonucleic acid

DMF- Deionized Formamide

EC- Extra-cellular

EDTA- Ethylenediaminetetraacetic acid

ERG- Electroretinograms

EtOH- Ethanol

GWS – Genome Wide Scan

Hz- Hertz

kD- KiloDalton

LOD – Logarithm of the ODds

Mb – Megabases

$MgCl_2$- Magnesium Chloride

mM- milliMolar ($1x10^{-3}$)

NSD- Non-Syndromic Deafness

List of Abbreviations and Symbols (cont'd)

OMIM- Online Mendelian Inheritance in Man

PCDH15 – Protocadherin15

rpm- revolutions per minute

RP- Retinitis Pigmentosa

SNHL- Sensory Neural Hearing Loss

SNPs- Single Nucleotide Polymorphisms

STR- Short Tandem Repeats

Taq- *Thermus aquatious* DNA Polymerase

TBE- Tetrabromoethane

UCSC- University California Santa Cruz

USH – Ushers syndrome

UV- Ultra-violet

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Chapter 1: Introduction

Overview of Hearing Loss

Hearing loss is a common sensory disorder and is prevalent in many populations. In the United States and the United Kingdom for example, 1 out of every 1000 live births is affected with hearing loss, and an equal frequency of children become deaf before the age of maturity (Bitner-Glindzicz 2002, Morton 2000). Furthermore, the prevalence of hearing loss increases dramatically with age: 10% of the population is affected by age 65 and 50% by age 85 (Liu et al. 2007).

The causes of hearing loss are categorized as environmental (25%), genetic (50%) and unknown etiology (25%) (Willems 2000). However, the true percentage of hearing loss caused by genetic factors is greater than 50%, as the majority of hearing loss cases of unknown etiology are expected to be caused by genetic factors. As well, the environmental cases of hearing loss are suspected to be influenced by genetics factors (Keats and Berlin 2002; Nance 2003). Of the deafness caused by genetic factors, 70% of the cases are non-syndromic, meaning the hearing loss occurs without any other symptoms, and 30% are syndromic cases, meaning the hearing loss is accompanied by maladies such as blindness (Ushers syndrome), progressive loss of kidney function (Alport syndrome) or branchial and renal anomalies (Branchio-oto-renal syndrome) (Keats and Berlin 2002). Among the explained cases of non-syndromic hearing loss, 88% of the identified genes are autosomal recessive, 11% are autosomal dominant, and 1% are either mitochondrial or X-linked genes. The most common genetic-type of deafness is autosomal recessive, non-syndromic deafness. Environmental causes of deafness include exposure to sustained high sound pressure levels (>90decibels, dB, head trauma,

ototoxic medication (ie. aminoglycoside antibiotics), prematurity, neonatal hypoxia, low birth weight, severe neonatal jaundice, prenatal infection (eg. rubella, CMV) and postnatal infection (eg. cytomegalovirus, meningitis) (Willems 2000; Bitner-Glindzicz 2002). Figure 1.1 shows the relative percentages of each cause of hearing loss.

Five factors are considered when describing the type of hearing loss. These are: age of onset, which frequencies are affected (ie. low, middle or high), the degree of hearing loss (measured in decibels), biological cause (ie. conductive, sensorineural or mixed) and configuration (ie. unilateral, one ear or bilateral). Table 1.1 outlines the sub-classifications within these five factors.

Figures 1.2 and 1.3 are examples of audiographs taken from subjects used in this research study. An audiograph is a graph generated from a standard hearing test. The y-axis scale measures sound intensity, in units of decibels (dB) which increases logarithmically. The normal threshold for hearing is 0dB, which is a barely audible whisper. Persons with hearing loss have a higher than normal hearing threshold, meaning the sound intensity must be greater than 0dB for them to hear. Persons with a mild degree of hearing loss can only hear at a sound intensity above 20dB, and persons with profound hearing loss cannot detect sound at all above >100dB (sound of a jet plane at ground level) (Table 1.2). The x-axis of the audiograph measures the frequency, or pitch, of a sound, in units of Hertz (Hz, number of cycles per second). Low pitch sounds have low frequencies (<500Hz), medium/middle pitch sounds have medium frequencies (500-2000Hz), and high pitch sounds have high frequencies (>2000Hz). Hearing loss is characterized by intensity (mild, moderate, severe, etc.) and by which frequency is

affected (low, middle or high). The two lines on the audiographs represent either ear and are distinguished by colour or line shape.

The individual who's audiogram is pictured in Figure 1.2 has been diagnosed with high frequency (>2000Hz), mild hearing loss (21-40dB) in both ears (bilateral). This type of hearing loss is common in older persons who lose their hearing as a result of presbycusis. Age-related hearing loss, or presbycusis, is caused by a degeneration of the ear's sensory cells, and is characterized by a mild to moderate hearing loss at higher frequencies (Friedman 2003).

The individual who's audiogram is pictured in Figure 1.3 has been diagnosed with profound hearing loss (>100dB), occurring across all frequencies and affecting both ears (bilateral). This type of hearing loss is a typical profile of hearing loss with a genetic cause because all frequencies are affected and the hearing loss occurs in both ears.

Genetic Contribution to Hearing Loss

Deafness is genetically heterogeneous, as most of the known genes cause the same type of deafness. As of May 2007, 121 non-syndromic deafness loci have been mapped and 47 genes have been identified (Hereditary Hearing Loss Homepage, 2006). Since the number of mapped deafness loci is more than twice the number of identified genes, there are at least another 50 non-syndromic deafness genes not yet described.

Twenty-three of the identified non-syndromic genes cause autosomal dominant deafness and twenty-one cause autosomal recessive deafness. One gene has been identified as causing X-linked deafness, and two genes have found to cause mitochondrial inherited deafness. Interestingly, four genes (*GJB2, GJB6, MYO7A* and

TMC1) have been found to cause both autosomal dominant and autosomal recessive deafness – the specific mutation determines the resulting mode of inheritance. Table 1.2 lists all the non-syndromic deafness genes identified to date (May 2007), as found on the Hereditary Hearing Loss Homepage.

Most of the non-syndromic recessive deafness genes were mapped using large consanguineous families from geographically or ethnically isolated populations such as Tunisia, India and Lebanon (Bonne-Tamir et al. 1997). Since Newfoundland has a number of large consanguineous families which segregate with hearing loss, and the population is a genetically isolated population, Newfoundland has great potential for novel gene discovery in the field of hearing loss.

As of May 2007, thirty syndromic genes for nine different syndromes have been identified to date (Hereditary Hearing Loss Homepage). A total of 13 loci and 9 genes have been mapped for Ushers syndrome - an autosomal recessive disorder characterized by hearing loss, visual loss and vestibular dysfunction (Ouyang et al. 2005). Ushers syndrome (or Ushers) is divided into three subtypes, classified by severity: a person with Ushers Type I (USH1), the most severe subtype, has congenital deafness, retinitis pigmentosa (RP) and early onset vestibular dysfunction; Ushers Type II (USH2) has moderate/severe hearing loss, no vestibular dysfunction, and early onset retinitis pigmentosa, and Ushers Type III (USH3), the least severe, has normal hearing and vision at birth, progressive development of both retinitis pigmentosa and hearing loss, and variable vestibular dysfunction (Online Mendelian Inheritance of Man, 2007). Ushers syndrome accounts for more than 50% of individuals who are both deaf and blind (Ouyang et al. 2005).

A number of deafness genes cause both non-syndromic and syndromic hearing loss. Ushers syndrome Type 1D (USH1D) is caused by a homozygous mutation in *CDH23* and Ushers syndrome Type 1F (USH1F) is caused by homozygous mutation in *PCDH15* (Online Mendelian Inheritance of Man, 2006). Allelic mutations in both *PCDH15* and *CDH23* cause non-syndromic autosomal recessive hearing loss as well (DFNB12 and DNFB23, respectively). It is predicted that missense mutations cause milder phenotypes (such as presbycusis and non-syndromic hearing loss) and null alleles cause more severe phenotypes (such as the Ushers syndromes) (McHugh et al. 2006). Evidence for this theory of genotype-phenotype correlation has been reported for the Wolframin gene (*WFS1*). Wolfram syndrome is a recessively inherited syndrome, characterized by deafness, diabetes-mellitus and optic atrophy. Interestingly, single mutations in *WFS1* have been found which cause isolated autosomal dominant deafness. Mutations in *WFS1* which are protein-inactivating cause Wolfram syndrome and mutations clustered in the C-terminus of the protein and do not alter the protein function cause isolated deafness (McHugh et al. 2006). Evidence for this genotype-phenotype association has also been confirmed in *CDH23* (Astuto et al. 2002).

Background on Newfoundland Population

History of the Newfoundland Demographic

The island of Newfoundland, part of the Canadian province Newfoundland and Labrador, is the most easterly landmass in North America, and is separated from Europe only by the Atlantic Ocean. Historically, Newfoundland was discovered in 1497 by the European explorer Giovanni Gabotto (John Cabot) (although the Vikings from Norway and Native Indians were living here before this date). A report of Cabot's voyage to Newfoundland, written by Raimondo di Soncio of London to the Duke of Milan states:

> "...The sea is full of fish which are taken not only with the net, but also with a basket, in which a stone is put so that the basket may plunge into water...And the Englishmen, his [(Cabot's)] partners, say that they can bring so many fish that the kingdom will have no more business with Islanda [Iceland], and from this country there will be a very great trade in the fish they call stock fish..." (Prowse, 2002, p.11)

Reports such as this circulated throughout Europe during the 16th century, attracting many Europeans to Newfoundland. Consequently, people from England, Scotland, Ireland, France and Portugal traveled to Newfoundland to harvest from the richest fishing grounds in the world. However, when the fishing season was over, the Europeans returned back home since permanent residency in Newfoundland was prohibited. And if this did not drive the fishermen away, the harsh Newfoundland winter and lack of basic supplies would have made a year-long stay a very unwelcoming option. Eventually however, the European fishermen adapted to Newfoundland's climate, and they began to over-winter, remaining on the island for years at a time (Hancock 1989; Bennett 2002; Poole and Cuff 1994).

By the early 1800s, the European fishermen began to colonize Newfoundland bringing their families to the island, with no intension of returning to Europe. The

families of these European fishermen are the founders of the Newfoundland population. The majority of these founders emigrated from either Southwest England or Southeast Ireland. Once in Newfoundland, these people were brought to small, unpopulated inlets along Newfoundland's coast in groups of one or two families. These settlements were very isolated because travel to other parts of the island was difficult and dangerous. This isolation led to intermarriages (interbreeding) amongst the settler families, which carried on for many generations. Although these inlet communities are no longer geographically isolated, the years of inbreeding have left a significant impact on Newfoundland's gene pool. This impact is reflected in the high rate of certain autosomal recessive diseases, which include hereditary deafness, Newfoundland rod-cone dystrophy, and Bardet Biedel Syndrome (Newhook et al. 2004; Bennett 2002; Eicher et al. 2001; Ives et al. 1991; Poole and Cuff 1994; Hancock 1989).

High Occurrence of Consanguinity in the Population

It is well documented that children of parents who are related have a higher percentage of homogenous alleles than children of unrelated parents. Theoretical calculations predict a level of 6.25% homozygosity in the genome of children from first cousin marriages (Arab et al. 2004), but empirical calculations suggest a higher level of homozygosity. For example, in a population with a long history of interbreeding, a level of 11% homozygosity was found in the genomes of children whose parents are first cousins (Woods et al. 2006).

Increasing the level of homogeneity increases the probability of inheriting two homozygous mutations resulting in a recessive disease. The effect of inbreeding on

frequency of recessive diseases has been shown in diseases such as the hemoglobinopathies and in deafness (Arab et al. 2004). It is likely that inbreeding is also responsible for the high incidence rate of certain recessive diseases in the Newfoundland population including Bardet-Biedl Syndrome, Type 1 diabetes mellitus and child-onset severe deafness (Moore et al. 2005; Newhook et al. 2004; Ives et al. 1991). The table below compares the frequencies of these recessive diseases found in Newfoundland compared with reference populations. Although it is saddening that the numerous generations of inbreeding in the Newfoundland population has led to high incidence rates of particular recessive diseases, these large, consanguineous families hold great potential for novel gene discovery.

Prevalence of Three Recessive Diseases in Newfoundland Compared with Prevalence in Other Populations

Disease	**Frequency in Newfoundland Population**	**Reference**	**Comparable Population**	**Reference**
Bardet-Biedl Syndrome	5.6/100,000	Moore et al. 2005	0.63-0.8/100,000 (European)	Moore et al. 2005
Type I Diabetes	35.93/100,000 (Avalon Peninsula)	Newhook et al. 2004	24.5/100,000 (PEI) 10.1/100,000 (Montreal)	Newhook et al. 2004
Child-onset Severe Deafness	1.2-9.5/1000 (Southwest coast)	Ives et al. 1991	1/1000 (US population)	Morton, *Annals New York Academy of Sciences*

Newfoundland is a Founder Population

A founder population is a subpopulation originating from a larger group, which has been isolated due to factors such as geography, culture or religion. Founder populations are subject to the "founder effect", whereby alleles from the original population are subject to random genetic drift and are either overrepresented, underrepresented or absent in the founder population. As a result of this random genetic drift, founder populations may have an elevated incidence of particular genetic disorders yet rare cases of other genetic disorders. A high prevalence of a disease-causing mutation provides evidence that a population has experienced the predicted outcome of the founder effect. Since Newfoundland is a subpopulation of the larger European population, isolated from Europe by geography and has undergone expansion in relative isolation, it is considered a founder population.

A founder mutation is a pathogenic mutation in a founder population that is found in two or more unrelated families that originates from the same ancestor. A number of founder mutations have been identified in the Newfoundland population. These include mutations in *MSH2,* a gene causing hereditary non-polyposis colorectal cancer; an exon 8 deletion has been found in 5 different families (N=74 carriers) and an intron 5 splice site mutation (c.942+3A>T) has been found in 12 different families (N=151 carriers) (Stuckless et al. 2007). As well, M390R, a mutation in *BBS1* causing Bardet Biedal Syndrome, has been identified in 6 different families (Moore et al. 2005). This thesis also reports a founder mutation in the deafness gene *TMPRSS3* c.782+3delGAG, which was identified in two Newfoundland families. These are only a few of the many examples of founder mutations which have been identified in the Newfoundland population, providing

evidence that Newfoundland is a founder population which has been subject to the founder effect.

Identifying Founder Haplotypes

A founder mutation is confirmed by building a haplotype flanking the chromosomal area around the mutation. Haplotypes which include a founder mutation are called founder haplotypes. Founder, or ancestral, haplotypes are expected to be common in founder populations. Many founder haplotypes, segregating with founder mutations, have been identified in the Newfoundland population, including a *TMPRSS3* haplotype (deafness), an *MSH2* haplotype (hereditary non-polyposis colorectal cancer) and a *BBS1* haplotype (Bardet-Biedel Syndrome) (Stuckless et al. 2007; Moore et al. 2005).

Identifying Disease Genes

Determining Pattern of Inheritance in a Family

The first step of identifying a genetic cause for a disease is determining the disease pattern of inheritance. This is done by examining the affection (disease) status from as many family members as possible to identify a recognizable pattern of inheritance. In autosomal dominant diseases, affected children have affected parents, and all generations in the pedigree are affected. In autosomal recessive diseases unaffected parents have affected children and the disease may skip generations. Both sexes inherit the mutation equally. In the remaining modes of inheritance, the sexes are affected differently. In X-linked recessive inheritance, the majority of affected are males while in X-linked dominant inheritance, affected fathers will have affected daughters but unaffected sons. An affected mother with an X-linked dominant mutation will have one-

half sons affected and one-half of daughters affected. Y-linked is only male to male transmission, and mitochondrial inheritance passed on only through the maternal line (Griffiths et al. 2002).

In order to accurately identify the disease pattern of inheritance in a family, the cause of the disease for each affected individual must be carefully described. Although a number of affecteds in the same family does indicate a genetic disease, it is possible that some family members may have the disease due to a non-genetic cause. An affected family member who has a non-genetic phenotype is called a phenocopy. It is important to use affected family members with a suspected genetic cause for a genome wide scan (technique used to identify disease gene), as a single phenocopy may render the entire results erroneous.

Identifying Candidate Disease Genes

Once the disease pattern of inheritance has been confidently identified, the next step is to compile a list of possible candidate genes – genes with disease-causing potential. These include any known disease genes, especially any which had been previously identified in the studied population, and any genes which are known to play a role in the pathology of the disease. Selected candidate genes are sequenced in affected individuals to search for a disease-causing mutation.

However, if a candidate gene cannot be identified - meaning that all the identified candidate genes have been sequenced and no mutation was identified, or the list of possible candidate genes is too extensive - the next step in disease gene identification is a genome wide scan (GWS).

Explanation of Genetic Tools and Techniques

Genome Wide Scan and LOD Score

The goal of a genome wide scan is to determine if a disease locus is common amongst affected family members. The GWS is performed using a few hundred evenly spaced microsatellite markers and requires DNA of family members from every affected sibship, every generation (both affected and not) and from both affected and unaffected family members. A LOD score (Logarithm of the ODds) is generated for each microsatellite marker. The LOD score is the logarithm of the ratio of two probabilities: probability 1 - the marker and disease gene are linked (θ=0), and probability 2 - the marker and disease gene are not linked (θ=0.5). The LOD score is generated for various linkage distances, and the distance with the highest LOD score is taken as the most probable. A LOD score of 3 is generally considered to be significant; a LOD score of $\geq$3 means there is a high probability (1000:1) that the marker is linked to a disease gene (Twilliger and Ott 1999).

Use of Microsatellite Markers to Construct Haplotypes

Common throughout the genome are areas of tandem DNA repeats, called microsatellite sequences. These microsatellites range from dinucleotide repeats (eg. TA) to a six-base repeats (GAAGTC). If the repeat number of the microsatellite is polymorphic, meaning the tandem DNA repeat number is variable in the population, it can be used as a genetic marker. For example, the dinucleotide microsatellite TA is polymorphic if it is repeated twice in one person (TATA) and three times in someone else (TATATA). These microsatellite markers are useful for comparing DNA from two or

more individuals. Genotypes from these markers are used to construct haplotypes. Depending on the subjects that are selected for genotyping, the variant may or may not be polymorphic. If all genotyped subjects have the same variant, the marker is "non-informative" since it provides no differentiating information between the subjects. Conversely, if subjects have variants of a genotype, the marker is "informative".

If a significant LOD score is generated for one of the microsatellite markers, the chromosomal locus surrounding the marker with the high LOD score is mapped using additional markers. These markers are used to create a short, well-defined map in the area with the high LOD score. The genotypes from these markers are used to build haplotypes - blocks of linked alleles which are passed on intact from parents to offspring. If all affected family members share a haplotype (two haplotypes for autosomal recessive diseases) there is a good chance the disease gene resides within the boundaries of the haplotype.

To determine which gene within the haplotype is the disease-causing gene the process for identifying candidate disease gene is repeated (ie. identifying candidate genes and sequencing). If sequencing candidate genes does not identify a disease mutation, all genes in the identified haplotype region may need to be sequenced in order to identify a disease-causing mutation.

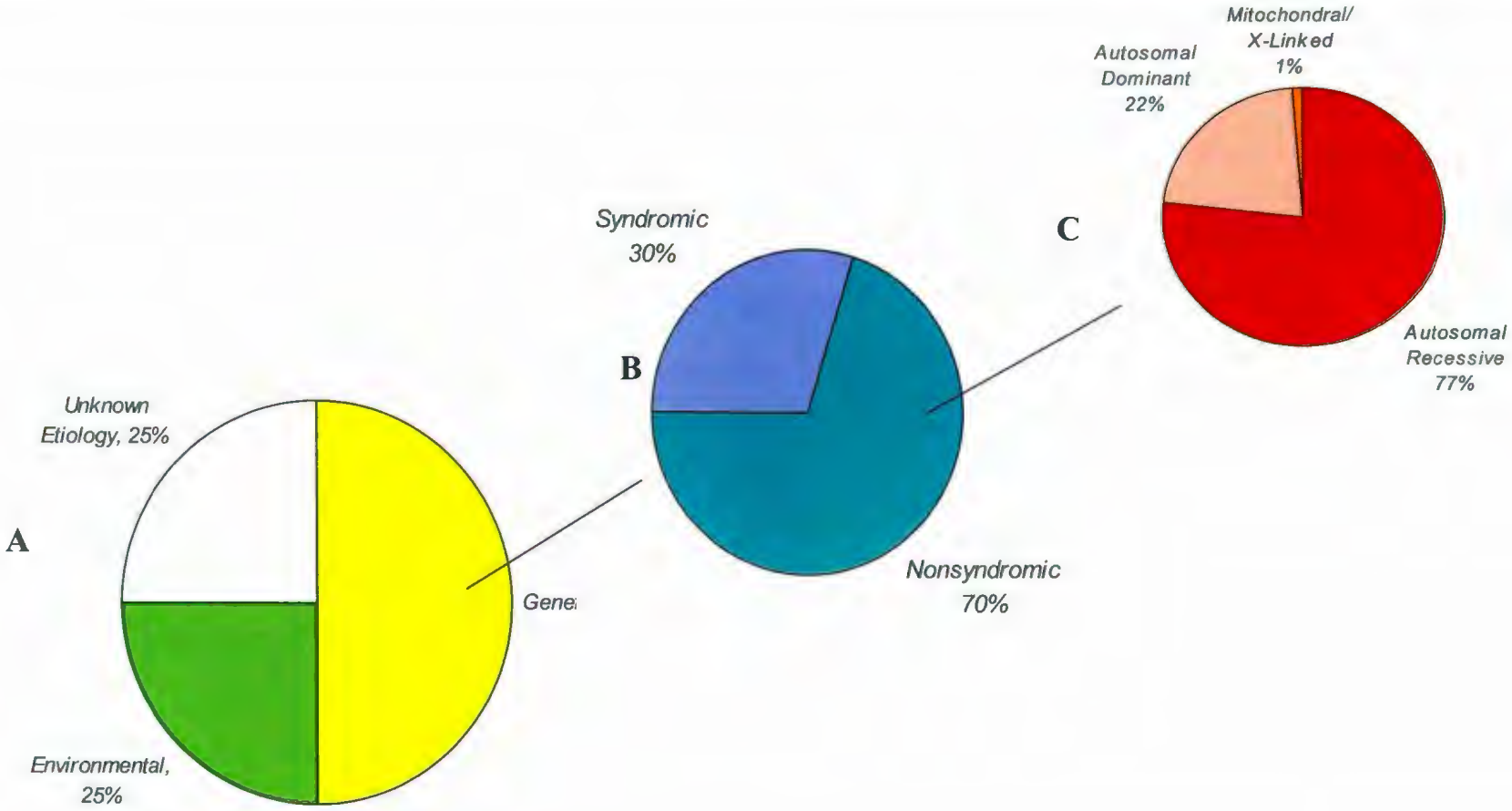


Figure 1.1 – Causes of hearing loss by percentage. Graph A shows the percentage contribution from each of the three major causes of hearing loss. Graph B is a sub-graph of Graph A; Of Genetic Hearing Loss, 30% is Syndromic and 70% is Non-syndromic. Graph C is a sub-graph of Graph B; Of Non-syndromic Hearing Loss, 1% is Mitochondrial or X-linked, 22% is Autosomal Dominant and 77% is Autosomal Recessive.

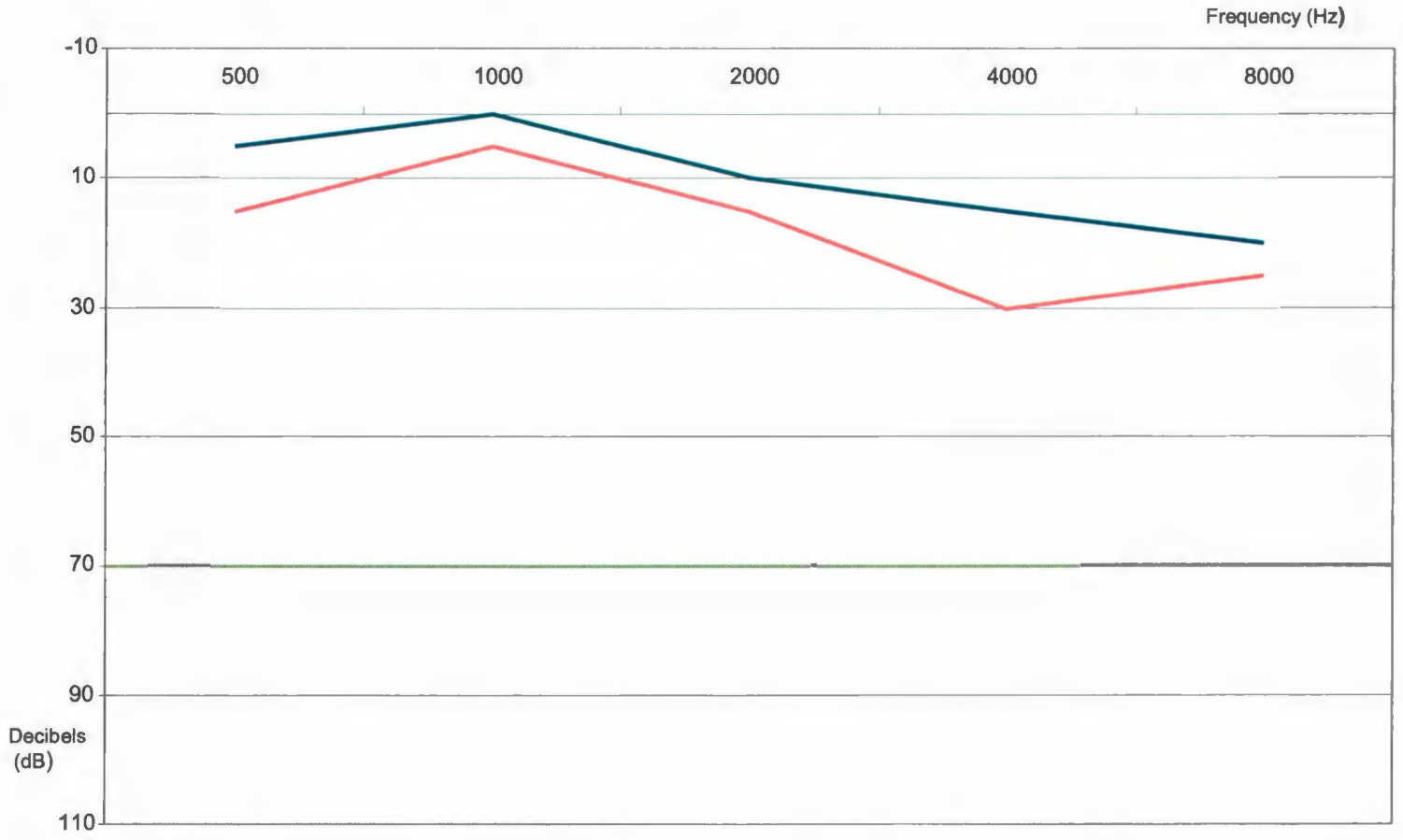


Figure 1.2 - Audiology chart of a hearing individual with mild hearing loss at the high frequencies. The different colours differentiate the two ears.

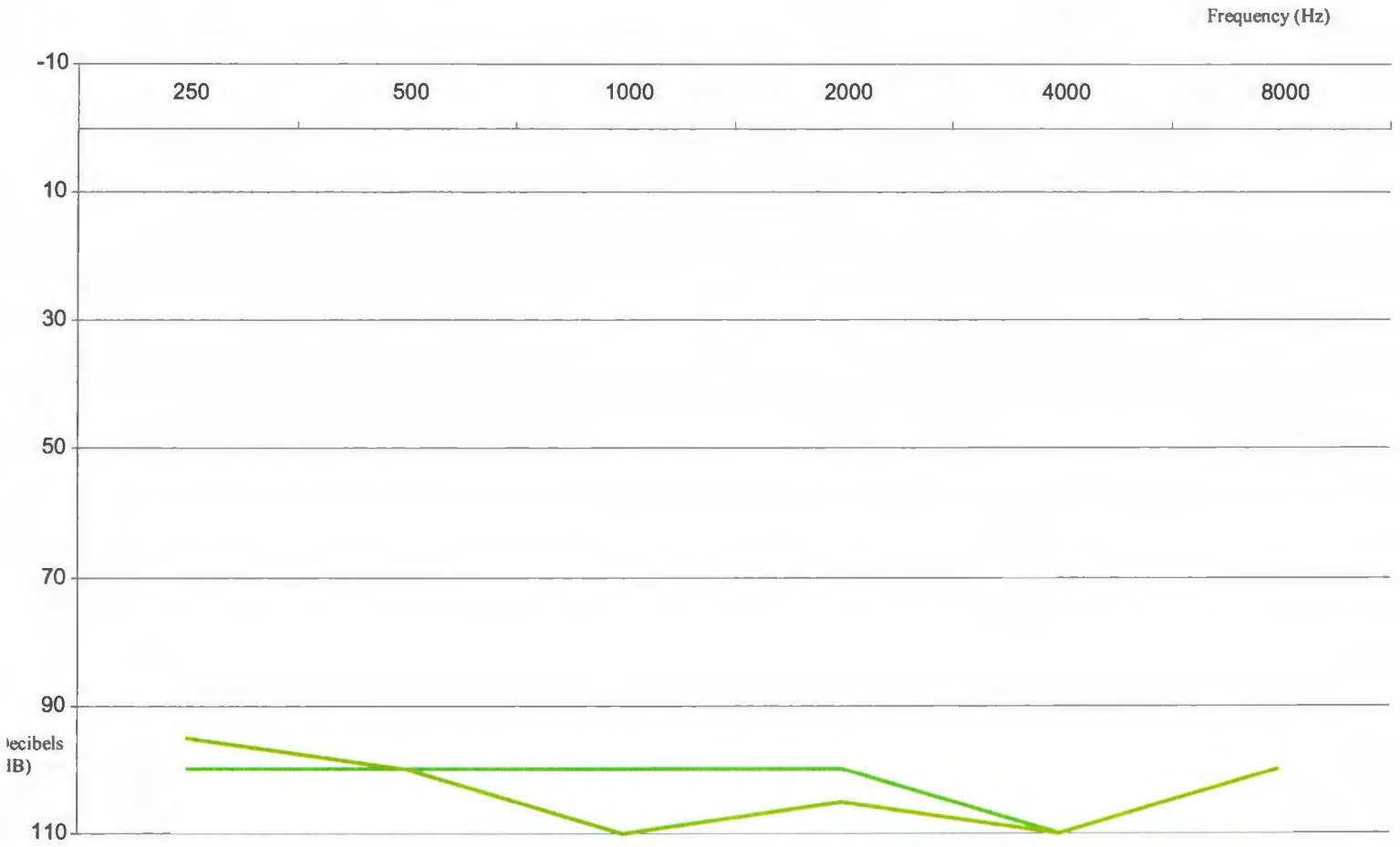


Figure 1.3- Audiogram of an individual with profound, bilateral, sensorineural hearing loss. The hearing loss is across all frequencies, occurs in both ears, and the individual cannot detect sound of <100dB. This type of hearing loss is the most severe, and is characteristic of hereditary hearing loss.

Table 1.1: Classification of Hearing Loss

Onset	
Birth	Congenital
Pre-lingual	Before Speech
Post-lingual	After Speech
Degree	
Normal hearing	Between 0-90dB
Mild hearing loss	Cannot detect sound below 21-40dB
Moderate	Cannot detect sound below 41-60dB
Mod Severe	Cannot detect sound below 61-80dB
Severe	Cannot detect sound below 81-100dB
Profound	Cannot detect sound <100dB
Configuration	
One ear	Unilateral
Two ears	Bilateral
Frequency	
Normal	20-20 000 Hz
Low Frequency	<500 Hz
Middle Frequency	501-2000 Hz
High Frequency	>2000 Hz
Biological Cause	
Sensorineural	
Conductive	
Mixed	Both sensorineural and conductive

Table 1.2: Non-Syndromic Deafness Genes Identified to Date (Hereditary Hearing Loss Homepage, April 2007)

Autosomal Dominant (AD) Autosomal Recessive (AR) X-linked (X)	Gene Name
AD	*CRYM*
AD	*DIAPH1*
AD	*GJB3*
AD	*KCNQ4*
AD	*MYH14*
AD	*DFNA5*
AD	*WFS1*
AD	*TECTA*
AD	*COCH*
AD	*EYA4*
AD	*COL11A2*
AD	*POU4F3*
AD	*MYH9*
AD	*ACTG1*
AD	*MYO6*
AD	*TFCP2L3*
AD	*MYO1A*
AR	*MYO15*
AR	*SLC26A4*
AR	*TMIE*
AR	*TMPRSS3*
AR	*OTOF*
AR	*CDH23*
AR	*STRC*
AR	*USH1C*
AR	*TECTA*
AR	*OTOA*
AR	*PCDH15*
AR	*TRIOBP*
AR	*CLDN14*
AR	*MYO3A*
AR	*WHRN*
AR	*ESPN*
AR	*MYO6*
AR	*TMHS*
X	*POU3F4*
AR/AD	*GJB2*
AR/AD	*GJB6*
AR/AD	*MYO7A*
AR/AD	*TMC1*

Chapter 2: Family A

Introduction

Family History

Family A is a large Newfoundland family from a small isolated community on the Southwest coast of Newfoundland. The complete pedigree documents 150 people, extends back six generations and reports four cases of consanguinity. The names and dates in the pedigree suggest the founders of this family may have also been the founders of the community. Twelve Family A members have been diagnosed with autosomal recessive, non-syndromic hearing loss which ranges in degree from mild to severe-profound. A summary of the audiology reports for twenty family members can be found in Table 2.1. A partial pedigree of Family A is shown in Figure 2.1.

Previous Research on Family A

Identification of a Deafness-Associated Haplotype

Previous work on Family A was conducted to determine which gene(s) was responsible for the family's deafness. A genome-wide scan (GWS) was performed on 32 Family A members. A marker which has a LOD score 3.0 or greater indicates a high probability the disease of interest segregates with the marker. A significant LOD score (Logarithm of the ODds) of 3.96 was generated for marker *D10S196*, located on chromosome 10q22 (Figure 2.2) (Young, *personal communication*). In order to narrow the critical region, two markers flanking either side of *D10S196* were typed in 16 Family A members (Figure 2.3). The alleles at the proximal marker *D10S208* did not segregate with either allele at *D10S196,* but an allele at the distal marker *D10S1652* did segregate with an allele at

D10S196 in 13 of the typed family members. It was determined that a disease haplotype must be distal to *D10S196.* The chromosomal area in this identified region was fine-mapped with additional markers and a common haplotype of 16.5 Mb was identified in 22 family members. A list of markers used for fine-mapping is found on Table 2.2. Figure 2.4 is a partial pedigree of Family A and displays the haplotypes which resulted from fine-mapping the area of interest on chromosome 10. All persons who carried two copies of the (yellow) haplotype were deaf, including T-10 and T-14 in sib-ship 3, T-16 in sib-ship 4, and T-7 and T-9 in sib-ship 5. Since this haplotype segregated with deafness, it was classified as a deafness-associated haplotype. The upper boundary of this haplotype can be clearly defined because the marker *D10S196* is informative. However, the marker at the lower boundary *D10S1646* is not as informative, and it is not clear whether or not the haplotype ends at this marker.

Identification of the Deafness-Causing Gene, *PCDH15*

Two deafness genes are located on chromosome 10, in the same region as the defined deafness-associated haplotype - *PCDH15* (protocadherin15) and *CDH23* (cadherin23). The *PCDH15* is located in the middle of the deafness-associated haplotype (55.3Mb on chromosome 10). Consequently, this gene, located in the area with the significant LOD score, segregates with the deafness-associated haplotype. The *CDH23* gene is located just outside the deafness-associated haplotype (72.9 Mb on chromosome 10). Although the *CDH23* gene is adjacent to the deafness-associated haplotype, it does not always segregate with deafness. Since the *PCDH15* gene segregated with the deafness-associated haplotype consistently, it was selected for full sequencing.

The Protocadherin15 gene is composed of 33 exons, spans 1.6 Mb on chromosome 10q21.1 and codes for 1955 amino acids. The start codon is in the middle of the second exon at 396bp and the stop codon is in exon 33 at 6,263bp. Two deaf members in Family A (T-16, T-17) and two individuals with normal hearing (unaffected Family A member (T-23) and non-Family A member with Newfoundland ancestry) were sequenced all *PCDH15* exons and intron/exon boundaries. A SNP found in *PCDH15* exon 13, designated c.1583T>A, was suspected to be pathogenic because it was homozygous in the two deaf individuals and heterozygous or wild-type in the two hearing individuals. To determine whether the mutation segregated with deafness consistently, a number of additional Family A members were sequenced for the c.1583T>A variant, which determined that all family members homozygous for the SNP were deaf and those heterozygous were both deaf and hearing. The genotypes for each individual sequenced for the *PCDH15* mutation are given in Table 2.1 and are marked on the pedigree in Figure 2.4.

All cadherin proteins, including protocadherin15 (PCDH15) have a conserved 5-repeat calcium-dependant binding motif. The function of these binding motives is to allow PCDH15 to interact with other proteins; a mutation in one of these conserved domains could disrupt the entire function of PCDH15. The *PCDH15* c.1583T>A SNP causes a missense substitution (Val528Ala) in one of these conserved binding motif and consequently was suspected to be a potentially pathogenic mutation.

The *PCDH15* c.1583T>A SNP explained the cause of deafness in five deaf Family A members homozygous for the mutation. Individuals T-10 and T-14 in sib-ship 3, T-16 in sib-ship 4, and T-7 and T-9 in sib-ship 5 are all homozygous for the *PCDH15*

mutation and have two copies of the deafness-associated haplotype. The deafness in these individuals is considered "solved". Deaf individuals T-19, T-20, T-1 and T-3 in sib-ship 2 and T-30 in sib-ship 1 are all heterozygous for the *PCDH15* mutation and carry a single copy of the deafness-associated haplotype; sequencing the full length *PCDH15* gene in selected family members did not identify any other potentially pathogenic SNPs. Individuals T-1 and T-3 were screened for mutations in connexin 26 (*Cx26)* and connexin (*Cx30*), the two most common deafness genes and for the three known Newfoundland deafness mutations (c.2146G>A in *WFS1* and c.207delC and c.782+3delGAG in *TMRPSS3*). These individuals did not carry any of the named mutations (Young, *personal communication).*

The previous research on Family A concluded that the *PCDH15* c.1583T>A mutation could explain the deafness in sib-ships 3, 4 and 5 but could not fully explain the deafness in sib-ships 1 and 2. It is of interest to note that the phenotype of the deaf individuals in sib-ship 2 differs from the other affected family members. It has been reported by researchers who have visited this family that the deaf siblings in sib-ship 2 can speak, which is indicative of a post-lingual hearing loss. By contrast, the audiology chart from T-16 in sib-ship 4 reports he has no speaking ability; the chart from T-9 in sib-ship 5 reports her onset of deafness was at 18 months (pre-lingual). Since the deafness phenotype in sib-ship 2 differs in its onset, the affecteds in this sib-ship may have a different type of deafness and a different genetic cause. It is also possible that the deafness in this sib-ship may not have a genetic cause and these siblings may be phenocopies. Environmental causes include severe neonatal jaundice, rubella, cytomegalovirus (CMV) and meningitis (Willems 2000, Bitner- Glindzicz 2002).

Digeneic Inheritance Between *PCDH15* and a Second Deafness Gene, *CDH23*

Zheng et al. (2005) reported a human case of Ushers syndrome (deafness, blindness) which was caused by the interaction of single mutations in *PCDH15* and *CDH23* (c.5601delACC/+ in *PCDH15* and c.193delC/+ in *CDH23).* Additionally, the same study reported double heterozygous mice presented with non-syndromic deafness. Two single mutations in two different genes which interact to produce a disease phenotype and segregate in an autosomal pattern of inheritance is called digeneic inheritance (Reiners et al. 2006).

The *PCDH15* c.1583T>A mutation segregated with the deafness-associated haplotype carriers in Family A which could explain deafness for five family members from sib-ships 3, 4 and 5. Five affected family members from sib-ships 1 and 2 were heterozygous for the *PCDH15* mutation and their deafness could not be explained by *PCDH15* alone.

The deafness gene *CDH23* resides at 4.7 Mb outside the deafness-associated haplotype and does not segregate with the chromosomal area with the significant LOD score (identified by the genome-wide scan). However, in light of findings by Zheng et al. (2005) on the digeneic interaction between mutations in *PCDH15* and *CDH23,* and since a single mutation was previously identified in *PCDH15* in deaf Family A members, it was hypothesized that a single mutation in *CDH23* could interact with the *PCDH15* mutation which might explain the deafness in the unexplained sib-ships.

Goals for Family A Research

The goal of this research is to determine if *CDH23* (a known deafness/Ushers syndrome gene) contributes to the genetic etiology of the hearing loss in a large inbred Newfoundland family, Family A.

Objectives

1. Sequence *CDH23* in three deaf siblings (T-20, T-1, T-3) from sib-ship 2 and one hearing relative (T-5), all heterozygous for the *PCDH15* c.1583T>A mutation. Determine whether if any of the *CDH23* SNPs are pathogenic.
2. Use the informative *CDH23* SNPs to construct *CHD23* intrageneic haplotypes – (haplotypes composed of variants from the one gene). Determine whether the deaf siblings in sib-ship 2 share a common *CDH23* intrageneic haplotype.

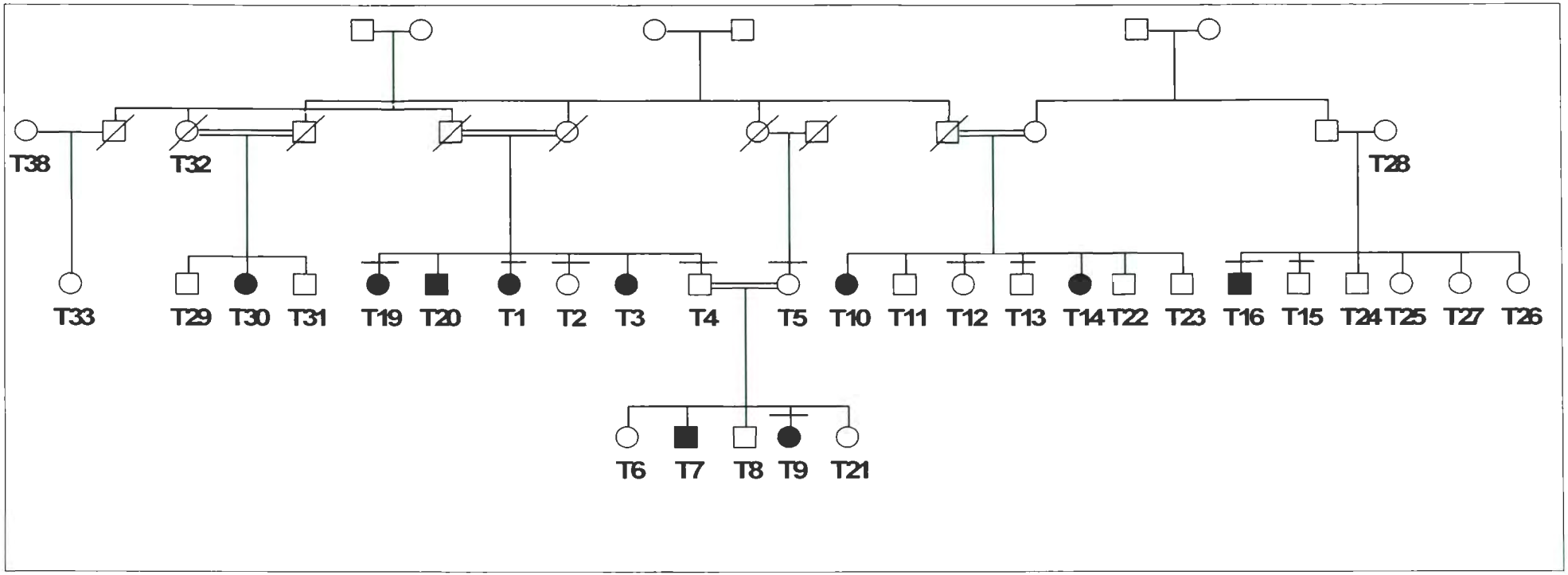


Figure 2.1 – Partial pedigree of Family A showing family members affected with bilateral, non-syndromic, sensorineural hearing loss (black symbol). Family members with unknown or non-genetic types of hearing loss are not identified in this pedigree. The horizontal line across the top of the symbol means an audiology report is available for the individual.

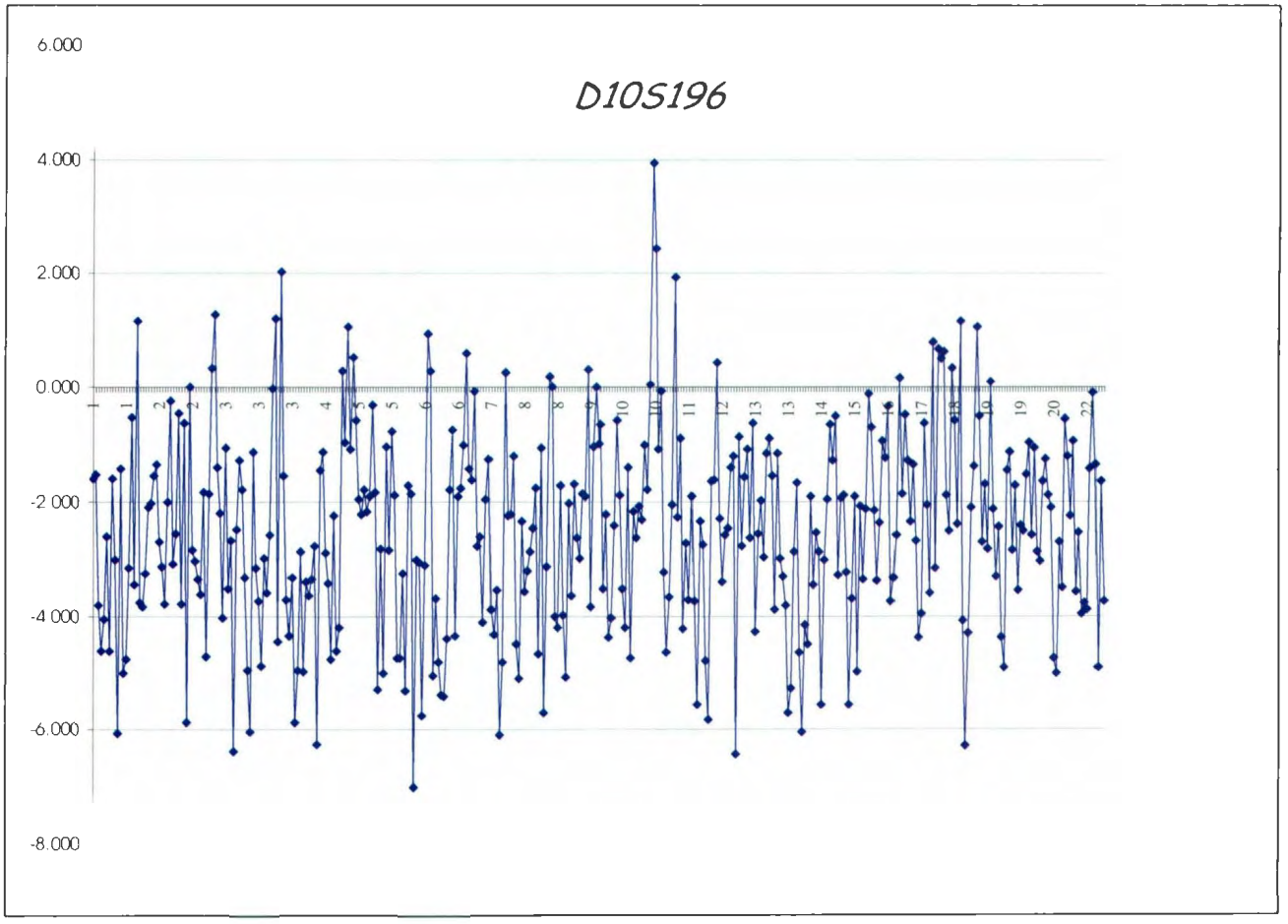


Figure 2.2: Plot of all LOD scores across the genome for Family A (Young, personal communication). The numbers on the x-axis represent the autosomal chromosomes (ie. 1= chromosome 1). The y-axis gives the LOD scores (axis on a logarithmic scale). The points represent the maximum LOD score for each marker. A LOD score >3.0 is significant, meaning there is a high probability (1:1000) that the disease trait is linked to the marker. In this case, the disease trait is deafness. The maximum LOD score on this chart is from marker *D10S196* on chromosome 10, with a LOD score of ~ 4.0, meaning there is a high probability (1:10,000) that deafness segregates with marker *D10S196*.

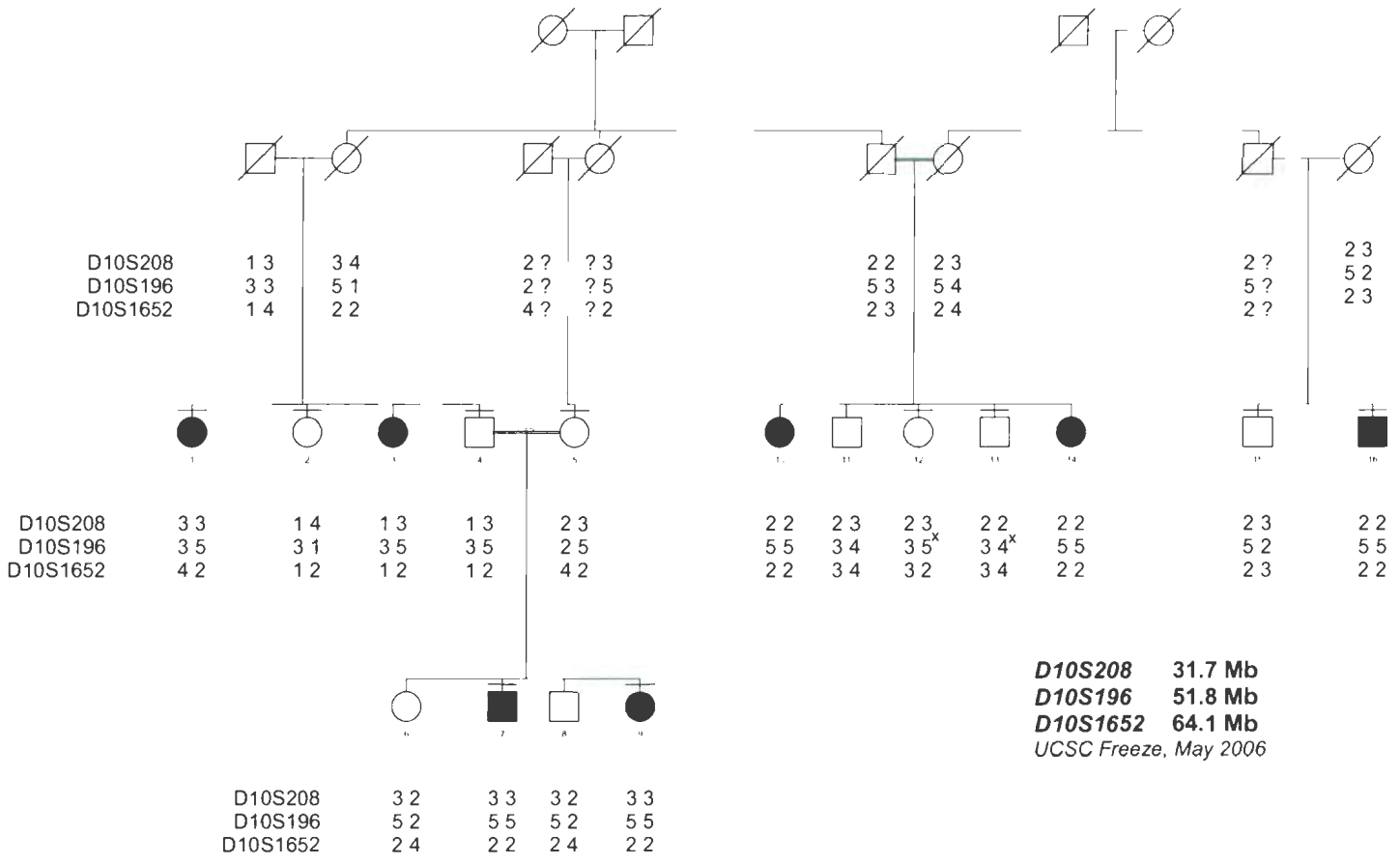


Figure 2.3: Markers used to narrow the critical region around marker *D10S196* (Young, personal communication) Markers flanking either side of *D10S196* were used to determine if the haplotype was proximal or distal to this marker.

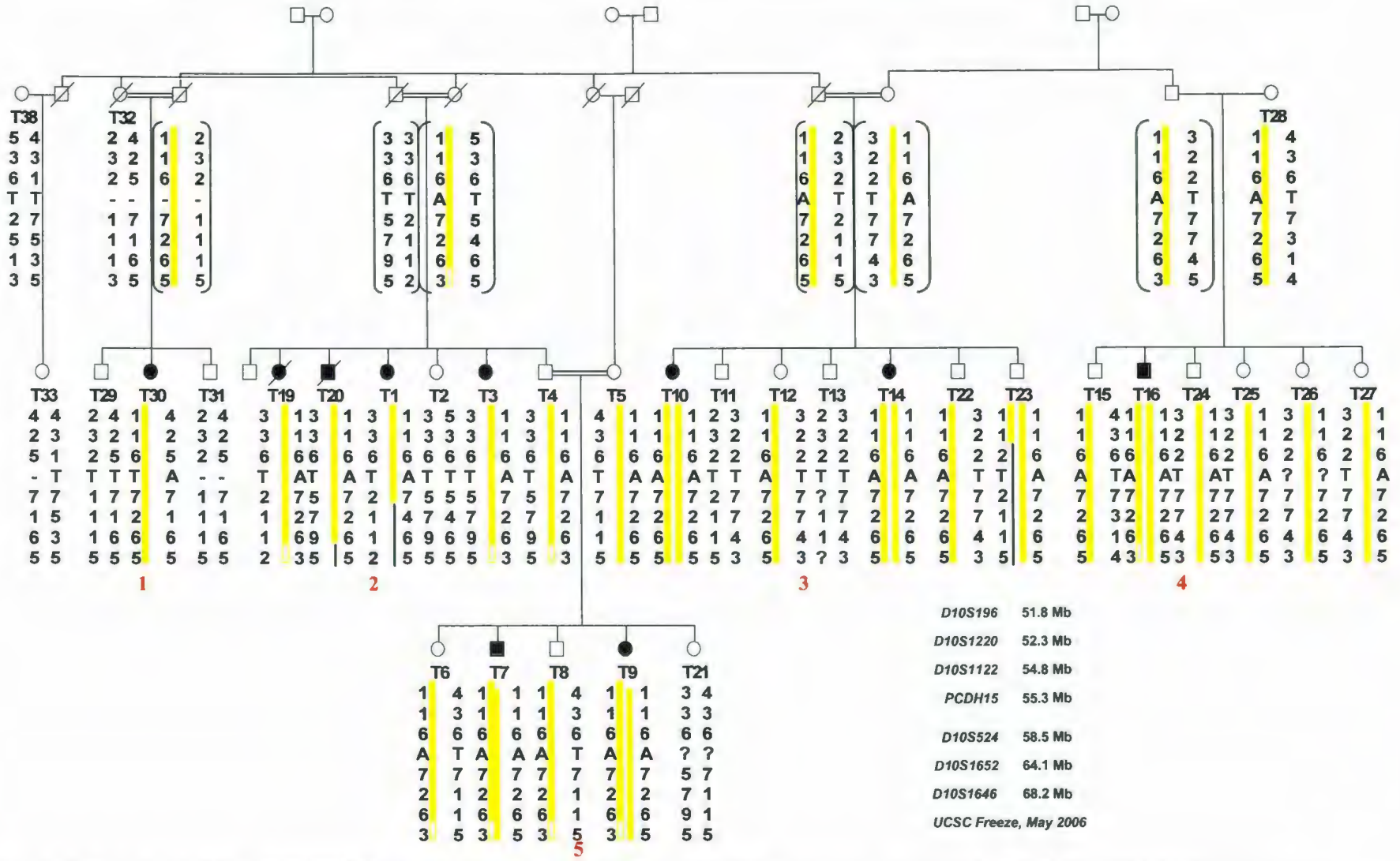


Figure 2.4: Carriers of the *PCDH15* deafness-associated haplotype (Young, personal communication). The *PCDH15* deafness-associated haplotype is highlighted by the yellow bar. Red numbers represent sib-ships. The legend is list of microsatellite markers used to map the haplotypes (University of California Santa Cruz, 2006). Some haplotypes have been inferred (brackets).

Identification of the *PCDH15* Deafness-Associated Haplotype
To accompany Figure 2.4

I. **The yellow haplotype represents the *PCDH15* deafness-associated haplotype, defined between markers *D10S196* and *D10S1646* and includes the *PCDH15* mutation c.1583T>A**. This *PCDH15* c.1583T>A mutation is potentially pathogenic.

II. **The *PCDH15* c.1583T>A mutation can explain the deafness in sib-ships 3, 4 and 5.** All deaf individuals in sib-ship 3 (T-10, T-14), sib-ship 4 (T-16) and sib-ship 5 (T-7 and T-9) are homozygous for the *PCDH15* c.1583T>A mutation. The deafness in these sib-ships is considered solved

III. **The *PCDH15* mutation by itself cannot explain the deafness in sib-ships 1 and 2.** The affecteds in sib-ship 1 (T-30) and in sib-ship 2 (T-19, T-20, T-1 and T-3) are heterozygous for the *PCDH15* mutation and carry a single copy of the *PCDH15* haplotype.

IV. **The *PCDH15* deafness-associated haplotype for T-20 has a suspected cross-over at *D10S524* or at *D10S1652*.** Since the allele at *D10S1652* is non-informative, the cross-over may have occurred at either of these markers. The full length deafness-associated haplotype has been defined in sib-ships 3, 4 and 5, providing evidence that T-20 has a recombinant *PCDH15* haplotype.

V. **The *PCDH15* deafness-associated haplotype for T-1 has a suspected cross-over at *D10S546*.** Similar for T-20, the *PCDH15* haplotypes defined in sib-ships 3, 4 and 5 provide evidence that T-20 has a recombinant *PCDH15* haplotype.

Figure 2.5- Genetic Map of Markers used to Construct the Deafness-Associated Haplotype on chromosome 10. Markers start from centromere. Markers selected from Genome Browser (University California Santa Cruz, 2006). Map also shows loci of *PCDH15* and *CDH23*.

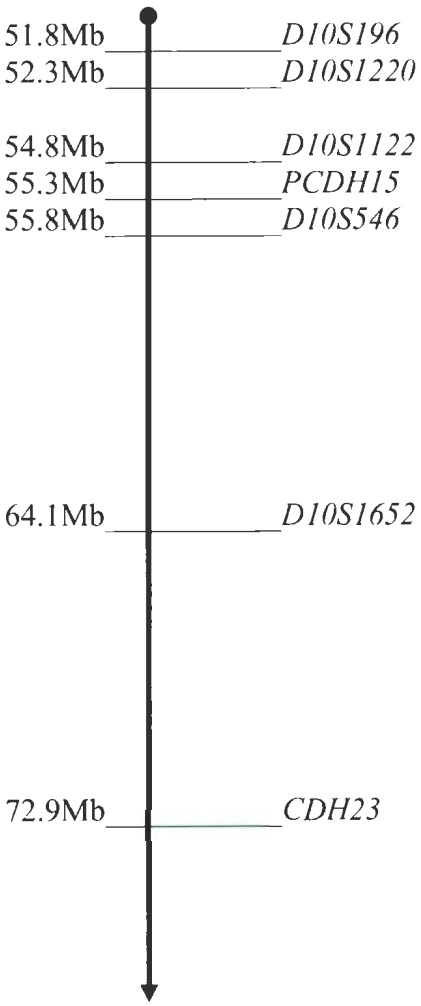


Table 2.1: Summary of Latest Audilogy Reports and *PCDH15* Mutation Status on Family A members

Pedigree#	Age (years)	Gender F=female M=male	Type of Hearing Loss	*PCHD15 c. 1583 T>A*	Age of Onset	Last Available Audiology (year)	Other Information ascertained from pedigrees and audiology reports
T1	65	F	Profound NSHL	A/T	n/avail	n/avail	Speaks a few words
T2	60	F	none	T/T	n/applic	1997	none
T3	56	F	NSHL	A/T	n/avail	n/avail	Not born deaf. Can hear loud noises
T4	77	M	none	A/T	n/applic	1997	none
T5	80	F	none	A/T	n/applic	1997	none
T7	39	M	Profound NSHL	n/a	n/avail	n/avail	none
T9	37	F	Moderate-Severe NSHL	A/A	18 months	1994	none
T10	59	F	NSHL	A/A	n/avail	n/avail	none
T11	80	M	none	T/T	n/applic	n/applic	none
T12	77	F	none	A/T	n/applic	1997	none
T13	43	M	none	T/T	n/applic	1997	none
T14	54	F	NSHL	n/avail	n/avail	n/avail	none
T15	63	M	none	A/T	n/applic	1997	none
T16	56	M	Profound NSHL	A/A	n/avail	1997	Surgery for growth in RE tunnel @ 22 years
T19	69	F	NSHL	A/T	n/avail	n/avail	Sick when young. HL may not be genetic
T20	-	M	NSHL	A/T	n/avail	n/avail	none
T22	57	M	none	A/T	n/applic	n/applic	none
T25	69	F	Mild-Moderate Right Ear only	A/T	n/avail	1997	none
T29	66	M	none	T/T	n/avail	n/avail	none
T30	58	M	NSHL	A/T	n/avail	n/avail	none

Note: n/avail = not available n/applic = not applicable NSHL= non-syndromic hearing loss

Table 2.2: Physical Location of Markers used to Create the *PCDH15* Deafness-Associated Haplotype and Locus of *PCDH15* and *CDH23*. The markers were taken from Genome Browser (University California Santa Cruz, 2006)

Marker/Gene	Location on 10q21-22 Dist from centromere (bp)	Type of Nucleotide Repeat	Heterogeneity
D10S196	51,812,274	Dinucleotide	0.79
D10S1220	52,348,388	Trinucleotide	n/a
D10S1122	54,839,814	Dinucleotide	0.78
PCDH15	55,251,058	n/a	-
D10S546	55,764,404	Dinucleotide	0.68
D10S1652	64,077,501	Dinucleotide	0.78
CDH23	72,869,595	n/a	-

Methods

Subject Recruitment

Family A members were recruited through the Newfoundland Provincial Genetics Program, initiated in 1988 by Dr. E Ives. Research participants were required to give informed consent, which granted researchers permission to access medical records and family history. A blood sample (for DNA) was taken from all participants who were able. This project was approved by the Human Investigations Committee (HIC), Research Ethics Board of Memorial University, Newfoundland and Labrador (# 01.186). Audiological tests were performed by a local physician to determine the type of hearing loss of affected individuals and to confirm normal hearing in non-affected persons. Physical exams (also performed by a local physician) were performed to determine whether affected persons were affected with other conditions (ie. syndromic hearing loss). Vision and vestibular functions were performed to test for Ushers syndrome.

CDH23 Sequencing Primers and Conditions

Both the forward and reverse strand of each *CDH23* exon (69) was sequenced to ensure the entire coding region was covered. The intron/exon boundaries were also sequenced as the primers are intronic. Primer sequences and specific PCR requirements for each exon can be found in Boltz et al. 2001 and in Appendix C.

DNA Preparation, PCR Thermo-cycling, and Electrophoresis

DNA was extracted from whole blood and diluted to 100ng/µl and was stored at 4^0C (performed by lab staff). Then 1µL of diluted (stock) DNA was added to 2.5µL 10X

PCR Buffer, 2.5μL dNTPs (2mM), 0.75μL $MgCl_2$ (50mM), 0.2 L Taq polymerase (5 U/μL), 16.05μL of distilled dH_20, 1.0μL of forward primer (10μM) and 1.0μL of reverse primer (10μM). 5.0μL of dH_20 was replaced with betaine to enhance PCR product yield when needed. The mix was then centrifuged briefly of which 25μL added to each well on the PCR plate, sealed and centrifuged briefly and was placed in the ABI thermo-cycler. Each primer pair had specific thermo-cycler conditions (Boltz et al. 2001 or Appendix C). The post-PCR products were run on a 1% agarose gel (1g agarose/1L TBE), stained with ethidium bromide, and viewed under UV light on the Kodiak Molecular Imaging system (Carestream Health, Inc. Rochester, NY, Version 4.01, 2005).

Preparation for ABI Cycle Sequencing

After re-suspending the Sephacryl 300, 300μL of the solution was added to each well on a silent screen plate. A plastic waste plate was placed underneath the silent screen plate to catch the flow through. The two adjacent plates were balanced and centrifuged at 3000rpm for 5 minutes and any flow-through in the plastic waste plate was discarded. The PCR products were then added to the wells on the silent screen plate and a clean PCR plate was added beneath the silent screen plate. The two adjacent plates were balanced and centrifuged 3000rpm for 5 minutes. The flow-through product collected in the PCR plate contained the Purified PCR products.

Successful amplified PCR products, visualized as bands of the appropriate size on an agarose gel, were prepared for sequencing using the following cocktail: 1μL of sequencing mix, 2μL 5X sequencing buffer, 2μL of either forward or reverse primer (1.6μM), 1μL of DNA template (Purified PCR product) and 14 μL of dH_20 (total volume

of 20μL). The mixture was then centrifuged briefly and the samples were denatured on the thermo-cycler (25 cycles of 96^0C at 1 minute, 96^0C for 10 seconds, 50^0C for 5 seconds, 60^0C for 4 minutes and held at 4^0C when completed). After denaturing, the samples could either be stored or continued to be prepared for sequencing. Stored samples were held at -4^0C for a maximum of 3-6 months.

1μL of purified PCR product, 5μL of 125mM EDTA and 65μL of 95% ethanol (EtOH) was added to each reaction well in a sequencing plate, and was vortexed and centrifuged. The mixture was precipitated for 1 hour to overnight, in a dark space at room temperature. After precipitation had occurred, the plate was centrifuged for 30 minutes at 3000g and subsequently was gently inverted to decant the ethanol. A folded paper towel and a plate carrier (used as a weight) were placed underneath the inverted plate and were collectively centrifuged very quickly to 200rpm. A volume of 150μL of 70% EtOH was added to each well and the plate was centrifuged again for 15 minutes at 3000g. Subsequently, the plate gently inverted to decant the ethanol. A paper towel and a plate carrier were placed underneath the inverted plate, which was then centrifuged until a speed of 200rpm was reached. The plate with the samples was left to dry in the dark for 15 minutes. 30μL of DMF (dimethylformaamide) was added to each well. The plate was then votexed and centrifuged briefly. The final mix was denatured at 95^0C for 2 minutes on a thermo-cycler (30μL of dH_20 was added to any empty wells in rows that had samples). Once denatured, samples were kept on ice until they were place in the ABI 3130 Automated Sequencer.

Automated Sequencing Using the ABI 3130

Automated sequencing was performed using the ABI Prism 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA). The raw sequence data were inspected manually (for quality) using Sequencing Analysis software. Good (clean) sequences were loaded into a program called Mutation Surveyor (Version 3.0, Softgenetics, State College, PA). Mutation Surveyor automatically detects DNA sequence variants in the sample DNA by comparing it to a reference gene sequence; the *CDH23* reference sequence was obtained from University California Santa Cruz genomic databank (University California Santa Cruz, 2006, Accession # AF312023). The informative SNPs were used to construct *CDH23* intragenic haplotypes. The haplotypes were constructed manually and were not computer generated. Mutation and protein naming of identified *CDH23* SNPs are taken from Human Genome Variation Society Homepage, 2007.

Results

The purpose of this study was to sequence *CDH23* in selected Family A members to determine if a mutation in this gene can explain deafness in sib-ships 2. Four family members were selected for sequencing, three of whom are deaf and one who has normal hearing (Figure 2.4, Persons T-20, T-3, T-1 and T-5, respectively). The three deaf individuals are from sib-ship 2 and the hearing individual is a first cousin to these siblings. All four sequenced individuals are heterozygous for the *PCDH15* c.1583T>A mutation. It was hypothesized that a single mutation in *CDH23* might explain the deafness in these *PCDH15* heterozygotes since the interactions between two single mutations in both *PCDH15* and *CDH23* has been demonstrated to cause both Ushers syndrome and non-syndromic deafness (Zheng et al. 2005).

The sequencing of *CDH23* in the four Family A members identified 45 different SNPs relative to the reference sequence. Each of the SNPs were categorized so to distinguish potentially pathogenic SNPs from polymorphic SNPs. Firstly the SNPs were classified as exonic and intronic. All exonic SNPs which occur in the coding region are potentially pathogenic as they have the ability to change the codon and alter the peptide. Intronic SNPs are pathogenic only if they occur in a splice site or if they create a cryptic site which could prevent exons from being translated and result in termination of the protein. These pathogenic intronic SNPs occur most commonly at the start or the end of an intron, and were not the case for any of the intronic SNPs identified in this project. Secondly, the exonic and intronic SNPs were classified as informative and non-informative. Informative SNPs are polymorphic, meaning alleles are different among the four sequenced individuals and non-informative SNPs are non-polymorphic meaning the

alleles do not vary amongst the four individuals. Thirdly, the exonic SNPs were classified as nonsynonymous and synonymous: a nonsynonymous SNP causes an amino acid change and a synonymous SNP does not. A summary table of the number of SNPs in each of these categories can be found in Table 2.3.

To test whether the *CDH23* intronic SNPs affected splicing, the BDGP splice-prediction program was used (Berkeley Drosophila Genome Project, 2004). The 9 informative intronic SNPs were analyzed to determine if the predicted donor or acceptor splice site differed between the reference sequence (without the intronic SNP) and a sequence which included the intronic SNP. No differences in splice sites were found, confirming that the 9 informative, intronic *CDH23* SNPs are not expected to be pathogenic.

The SNPs were deemed potentially pathogenic if they were exonic, informative and nonsynonymous. Using this criterion, eight of the 45 *CDH23* SNPs were potentially pathogenic. None of the eight potentially pathogenic *CDH23* SNPs were hypothesized to be disease-causing because no SNP was exclusive to the deaf individuals and therefore no SNP segregated with deafness. This conclusion was confirmed by the literature, which identified all eight SNPs as non-pathogenic. In a study done by Astuto et al. (2002), 69 probands with Ushers syndrome and 38 probands with recessive non-syndromic deafness were screened for mutations in the entire coding region of *CDH23*. The methods of heteroduplex analysis and single strand conformation polymorphism were used to screen for mutations, which identified numerous exonic and intronic polymorphisms. To determine which of these polymorphisms were pathogenic, a set of 96 genetically independent samples of mixed European ancestry were used as a control for both

methods. If a mutation was found in >1 control sample, it was considered non-pathogenic. The eight potentially pathogenic *CDH23* SNPs identified from this current research project are listed in the paper by Astuto et al. (2002) as non-pathogenic. The results from this project and from the research by Astuto et al. (2002) are in agreement, providing a level of certainty that none of the identified exonic, informative, non-synonymous *CDH23* SNPs are deleterious.

Informative SNPs (both exonic and intronic) were used to construct the *CDH23* intragenic haplotype. The large size of the *CDH23* gene (69 exons) proved useful for the haplotype analysis since a large number of SNPs were identified and therefore an informative haplotype could be constructed. Twenty-one *CDH23* SNPs were used to build this haplotype, which included 12 informative exonic and 9 informative intronic SNPs (genotypes are given in Table 2.4). Only informative SNPs are useful for constructing haplotypes because their genotypes can be used to differentiate between disease-associated haplotypes and non-pathogenic ones. Figure 2.5 shows the constructed *CDH23* intrageneic haplotypes segregating with the *PCDH15* mutation.

Individual T-3 is homozygous for all identified *CDH23* SNPs, meaning her mother and father have an identical *CDH23* haplotype. It is possible that T-3 has a hemizygous deletion, and she has a single copy of either the paternal or maternal *CDH23* haplotype. However, since her parents are first cousins there is a high possibility that they have passed on the same ancestral haplotype to T-3 (green haplotype, Figure 2.5).

The haplotypes in Figure 2.5 are constructed with the *PCDH15* mutation and the *CDH23* data. According to the *CDH23* data, the only expected cross-over in sib-ship 2 is

in T-3, between the *PCDH15* gene and the *CDH23* SNPc.1-88C>A. The previously ascertained *PCDH15* data for T-3 are insufficient to confirm or rule-out this cross-over.

The *CDH23* haplotypes for T-20 and T-1 are parental (non-recombinant) in both siblings and no cross-over between the *PCDH15* mutation and the *CDH23* haplotype was observed. These new *CDH23* haplotypes do not agree with the previously designed *PCDH15* haplotypes as a cross-over within the *PCDH15* haplotype (after the *PCDH15* mutation) was suspected in both T-20 and T-1 (Figure 2.4). Unfortunately, the original *PCDH15* data are not available to check for any potential genotyping errors. However, the original *PCDH15* haplotypes consisted of only 7 markers (only 6 are informative) and therefore may not be accurate. Three generations of DNA haplotypes and/or extensive, informative data from one or two generations is needed to accurately define haplotype boundaries. If only a single generation of haplotypes with limited data is available, the haplotype boundaries are based on best assumptions. In the sib-ship with the deaf heterozygotes (sib-ship 2, Figure 2.4), since no DNA is available from any other generation, it must be recognized that the recombinant haplotypes are based on best assumptions.

By contrast, the *CDH23* haplotypes are constructed based on 21 informative *CDH23* SNPs. Furthermore, the *CDH23* data has been checked numerous times for errors. Since the *CDH23* haplotyping data is more reliable and more complete then the *PCDH15* haplotypes, the *CDH23* haplotypes constructed in Figure 2.5 and are considered to be the most accurate.

The *CDH23* haplotypes constructed for the three deaf Family A members in sib-ship 2 show that each sibling has a different combination of paternal and maternal

CDH23 haplotypes, and that the three affecteds do not share a common *CDH23* haplotype. Since a shared *CDH23* mutation was not identified, a shared *CDH23* haplotype is not expected. The differing *CDH23* intrageneic haplotypes in sib-ship 2 support the conclusion that *CDH23* does not harbour a deafness causing mutation in Family A.

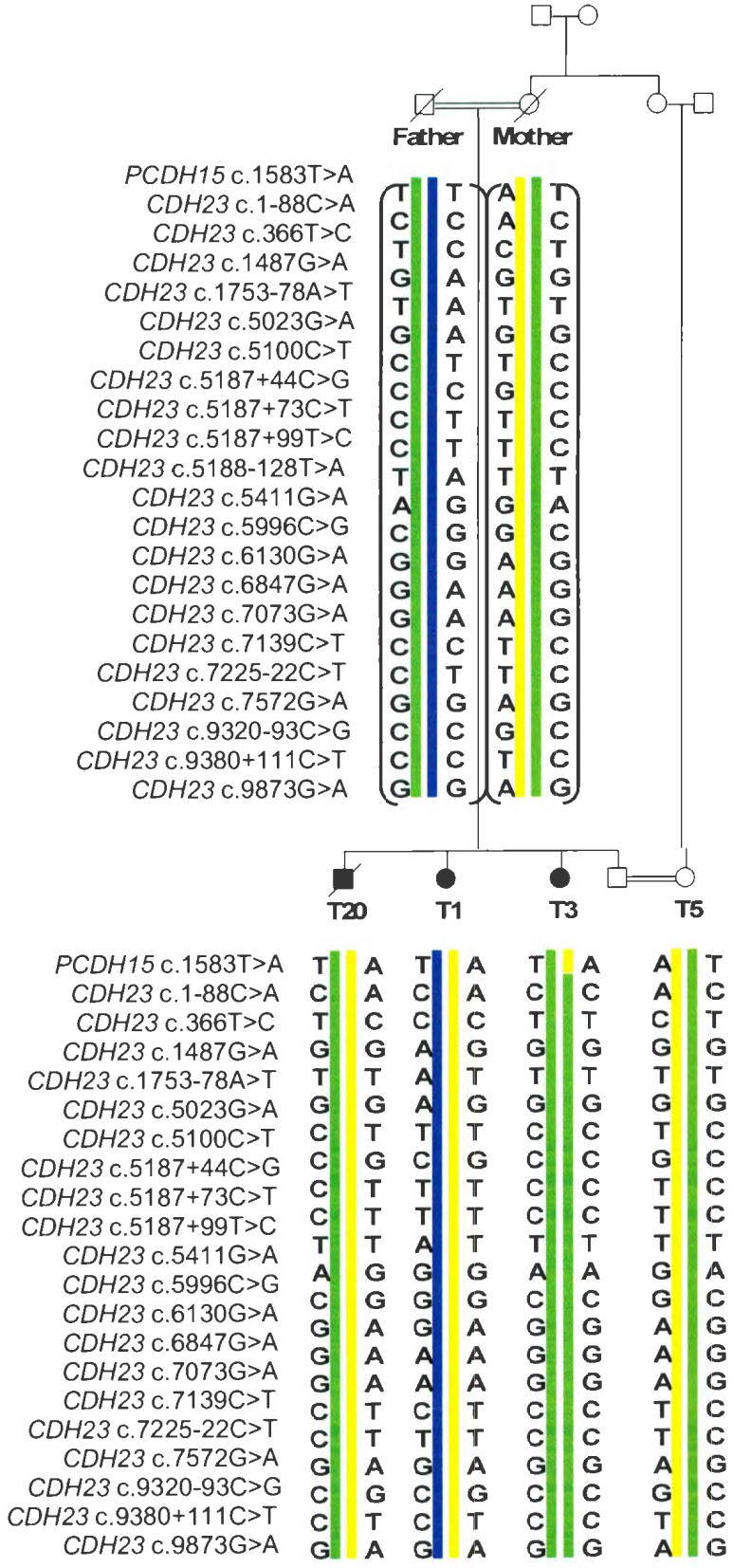


Figure 2.6- *CDH23* intrageneic haplotypes for selected Family A members. SNPs were identified by direct, automated sequencing of *CDH23* in these four individuals. The yellow bar (haplotype) is the *PCDH15* c.1583T>A deafness-associated haplotype. See next page for explanation.

Strategy used to create *CDH23* haplotypes

To accompany Figure 2.6

I. **Four members from Family A sib-ship 2 were selected for *CDH23* sequencing. All carry one copy of the *PCDH15* c.1583T>A mutation.** T-20, T-1 and T-3 are deaf and T-5 is hearing.

II. **Sequencing *CDH23* in these four Family A members identified 21 informative SNPs.** These SNPs were used to construct *CDH23* intragenic haplotypes for the four individuals.

III. **T-3 is homozygous for all identified *CDH23* SNPs** and therefore, her parents share a *CDH23* haplotype (green haplotype). It is possible that T-3 has a hemizygous deletion, meaning only one parental haplotype is present, but since the parents are known first cousins, sharing is expected.

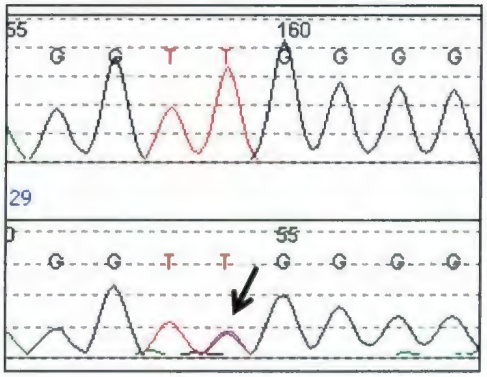
IV. **T-3 has a suspected cross-over between the *PCDH15* mutation and the *CDH23* haplotype on one chromosome**. The cross-over is suspected at this location because it results the least number of recombinant haplotypes within the sib-ship. Data from *PCDH15* haplotypes for T-3 neither supports nor refutes this recombination.

V. **T-20 has two parental haplotypes and no recombinations are suspected.** This is not in agreement with the previously defined *PCDH15* haplotypes in Figure 2.4, as T-20 has a cross-over at *D10S1652*. It is possible that there may be a mistake in the *PCDH15* data (original data not available), or that the *PCDH15* haplotypes could not be accurately constructed as not enough data was available. Since the *CDH23* data is more complete and more informative, it is therefore more reliable.

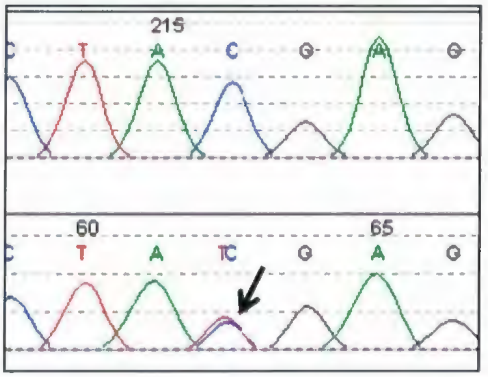
VI. **T-1 has two parental haplotypes and no recombinations are suspected.** This is not in agreement with the previously defined *PCDH15* haplotypes in Figure 2.4, as T-1 has a cross-over at *D10S524*. The same reasoning from T-20 can be applied to T-1, which concludes that the *CDH23* data is more reliable.

VII. **The haplotypes for the parents of sib-ship 2 are inferred, based on the haplotypes of the children.** The haplotyping data in sib-ship 2 is not enough to determine which parent segregates with the *PCDH15* mutation and the *CDH23* associated haplotype. However, the *PCDH15* mutation is known to segregate on the maternal side.

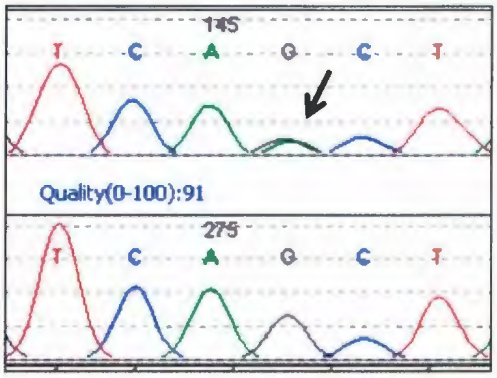
VIII. **T-5 data does not have enough familial data available to confidently ascertain phase.** However, as T-5 is a first cousin, some sharing can be expected.



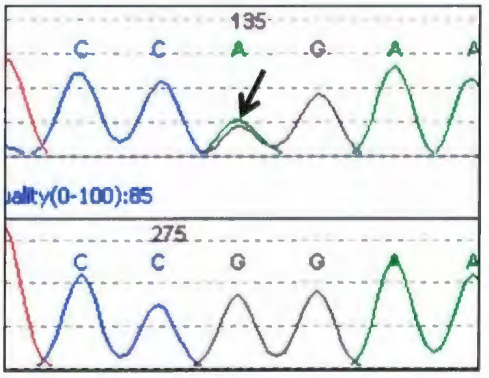
Exon 5 c.336T>C (Forward)



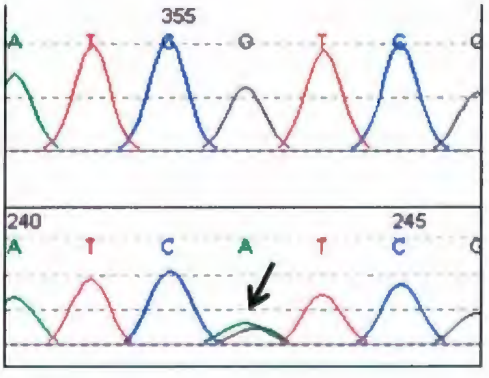
Exon 39 c.5100C>T (Forward)



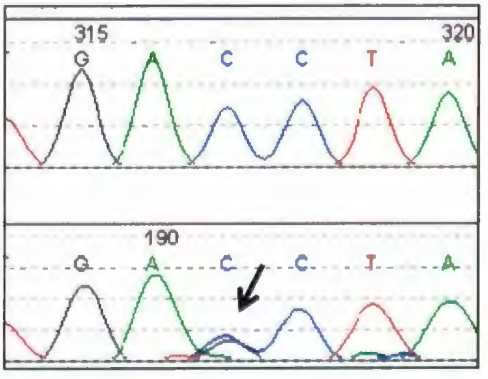
Exon 14 c.1487G>A (Reverse)



Exon 41 c.5411G>A (Reverse)

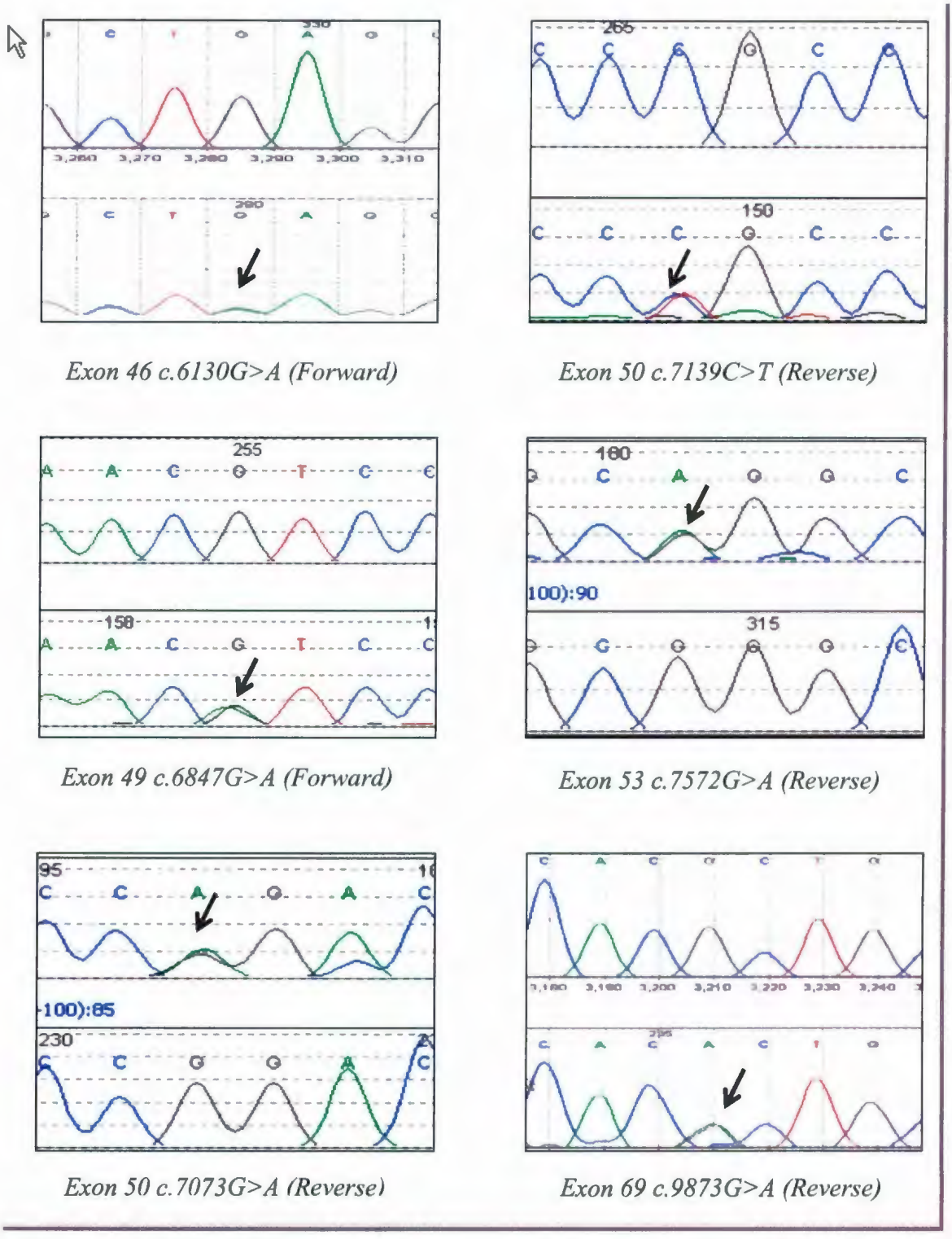


Exon 38 c.5023G>A (Forward)



Exon 45 c.5996C>G (Forward)

Figure 2.7: Electropherograms of the 12 exonic, informative *CDH23* SNPs identified by automated sequencing. The black arrow points to the SNP labeled in the caption. The reference wild-type sequence is located above or below the sample. Forward and reverse refers to whether the SNP has been identified by the forward or reverse primer.



Exon 46 c.6130G>A (Forward)

Exon 50 c.7139C>T (Reverse)

Exon 49 c.6847G>A (Forward)

Exon 53 c.7572G>A (Reverse)

Exon 50 c.7073G>A (Reverse)

Exon 69 c.9873G>A (Reverse)

Figure 2.7: Electropherograms of the 12 exonic, informative *CDH23* SNPs identified by automated sequencing. The black arrow points to the SNP labeled in the caption. The reference wild-type sequence is located above or below the sample. Forward and reverse refers to whether the SNP has been identified by the forward or reverse primer.

Table 2.3: *CDH23* SNPs Identified by Automated Sequencing

Total # *CDH23* SNPs Identified	**45**
Informative, Synonymous, Exonic SNPs	4
Informative, Non-synonymous, Exonic SNPs	8
Non-informative, Exonic SNPs	4
Total # Exonic SNPs Identified	**16**
Informative, Intronic SNPs	9
Non-informative, Intronic SNPs	20
Total # Intronic SNPs Identified	**29**

Table 2.4: Genotypes of Informative *CDH23* SNPs Identified by Sequencing
Mutation and protein naming is taken from Human Genome Variation Society homepage, June 2007. HL represents "Hearing Loss"

Exon	Nucleotide change	Theoretical Codon change	T-20 HL	T-1 HL	T-3 HL	T-5 Control
1	*c.1-88C>A*	5' untranslated region	CA	CA	CC	AC
5	*c.336T>C*	p.Val122Val	TC	CC	TT	CT
14	*c.1487 G>A*	p.Ser496Asp	GG	AG	GG	GG
16	*c.1753-78A>T*	intronic	TT	AT	TT	TT
38	*c.5023G>A*	p.Val1675Ile	AG	GG	AA	GA
39	*c.5100C>T*	p.Tyr1700Tyr	CC	TC	CC	CC
39	*c.5187+44C>G*	intronic	CG	GG	CC	GC
39	*c.5187+73C>T*	intronic	CT	CT	CC	TC
39	*c.5187+99T>C*	intronic	TC	CC	TT	CT
40	*c.5188-128T>A*	intronic	AT	TT	AA	TA
41	*c.5411G>A*	p.Arg1804Gln	GG	AG	GG	GG
45	*c.5996C>G*	p.Thr1999Ser	GC	CC	GG	CG
46	*c.6130G>A*	p.Glu2044Lys	GA	AA	GG	AG
49	*c.6847G>A*	p.Val2283Ile	GA	GA	GG	AG
50	*c.7073G>A*	p.Arg2358Gln	GA	AA	GG	AG
50	*c.7139C>T*	p.Pro2380Leu	CT	TT	CC	TC
51	*c.7225-22C>T*	intronic	CT	CT	CC	TC
53	*c.7572G>A*	p.Ala2524Ala	GA	AA	GG	AG
65	*c.9320-93C>G*	intronic	CG	CG	CC	GC
65	*c.9380+111C>T*	intronic	CT	CT	CC	TC
69	*c.9873G>A*	p.Trp3291Trp	GA	GA	GG	AG

Discussion

The objective of this study was to sequence the entire coding region and the exon/intron boundaries of the deafness gene *CDH23* to determine if it contributes to the unexplained cases of hearing loss in Family A. Previous work on this family identified a pathogenic mutation in the deafness gene *PCDH15* which explained the deafness in all family members homozygous for the mutation. However in sib-ships 1 and 2, four deaf family members were heterozygous for the *PCDH15* mutation; therefore, their deafness was not explained by the *PCDH15* mutation alone. Since the *PCDH15* mutation explained the deafness in Family A members who were homozygous for the mutation, it was expected that the *PCDH15* mutation may be partially responsible for the deafness in the heterozygotes. In a study published by Zheng et al. (2005), the interaction between single mutations in *PCDH15* and *CDH23* were found to be responsible for Ushers syndrome (deafness, blindness) in a human child, and for non-syndromic deafness in mouse models. As *CDH23* is located close to the previously defined deafness-associated haplotype which included *PCDH15*, it was hypothesized that a single mutation in *CDH23*, interacting with heterozygous *PCDH15* mutation, might explain the deafness in the unexplained siblings in sib-ship 2. Therefore, *CDH23* was selected for sequencing. Three deaf siblings from sib-ship 2 and a hearing cousin, all heterozygous for the *PCDH15* mutation, were selected for sequencing.

The study by Zheng et al. (2005), which reported single mutations in *CDH23* and *PCDH15* as causative for deafness in mice and Usher's syndrome in a single human case, is not unusual for Ushers genes. Digenic inheritance, or double heterozygotes, has been reported for other pairs of Ushers syndrome genes, including myosin VIIa and harmonin,

protocadherin15 and harmonin, and cadherin23 and harmonin (Reiners et al. 2006). Since the Ushers proteins are known to interact and are thought to function as a single complex unit, it is speculated that a dysfunction or absence in any one protein could disrupt the entire protein unit leading to the degeneration of the sensory epithelium of the inner ear, resulting in deafness (Reiners et al. 2005).

Protocadherin and Cadherin

Cadherins are a superfamily of genes with over 80 members, which includes classical cadherins, flamingo cadherins, protocadherins and seven-pass transmembrane cadherins (Zheng et al. 2005; Suzuki et al. 2000). Cadherins are cell adhesion molecules which form gap junctions between cells, allowing cell development and regulation, influencing cell behaviour, and controlling cell signaling and oncogenesis (Suzuki et al. 2000). Recently it has been reported that cadherins play a role in the migration of precerebellar neurons in the caudal hindbrain (Taniguchi et al. 2006). Characteristic to all cadherin proteins is a highly conserved cadherin-specific calcium binding motif. This binding motif is located in the extra-cellular domain and is repeated at least five times in all cadherin proteins. This motif is the site of cell-cell interaction, allowing the cadherins proteins to adhere to other intracellular proteins. The cytoplasmic domain (C-terminus) of cadherin protein is a highly conserved sequence, connected to its extra-cellular domain by a single trans-membrane segment (Suzuki et al. 2000).

Protocadherins belong in the "non-classical" family of cadherins. They are structurally unique because their cadherin-specific calcium binding motif is repeated more than five times and the extra-cellular domains of many protocadherins have no

introns (Suzuki et al. 2000). Protocadherins are required for the structure and organization of embryonal, neuronal and epithelial tissues during development and adulthood (Hampton et al. 2003).

The protocadherin15 protein (PCDH15) is expressed in the inner ear, in the cochlea and in the retinal photoreceptors. In the cochlea, it is expressed on the apical surface of the hair cells, in the supporting cells, the outer sulcus cells and the spiral ganglion cells. In the retina, PCDH15 is expressed in the inner and outer synaptic layers and in the nerve fiber layers (Ahmed et al. 2003). The human orthologue of *PCDH15* spans 1.6 Mb on chromosome 10q21.1, has 33 exons, 1955 amino acids, and has a molecular weight of 216kD. The start codon, located in the middle of exon 2, is at 396bp and the stop codon, located in exon 33 is at 6,263bp (Online Mendelian Inheritance of Man, 2006). Mutations in the *PCDH15* gene have been found mostly to cause syndromic deafness (USH1F), but at least one case of autosomal recessive non-syndromic deafness has been reported (Ahmed et al. 2003). The mouse model is the *Ames waltzer*, which presents with deafness, circling behaviour and head tossing due vestibular imbalance. The stereocilia (hair cells) in its inner ear are disorganized and there is degeneration of the inner ear neuroepithelia (Hampton et al. 2003).

Cadherin23 (CDH23) is a protein from the cadherin family. It is expressed in both sensory hair cells and in Reissner's membrane of the inner ear (Reiners et al. 2000). The protein has a short intracellular domain, a single-pass trans-membrane domain which contains a 27-repeat of the cadherin-specific calcium binding motif (Reiners et al. 2000). The *CDH23* gene has 69 exons, including two micro-exons, spans 300kB, and codes for 3354 amino acids (Bolz et al. 2001). Mutations in *CDH23* causing non-syndromic and

syndromic deafness have been well documented (deBrouwer et al. 2003, Bolz et al., 2001).

SNPs Identified by Sequencing *CDH23*

The deafness gene *CDH23* was sequenced in four Family A members to determine if a mutation in this gene contributed to deafness in the deaf individuals which could not be explained by the *PCDH15* mutation. A total of 45 distinct *CDH23* SNPs as compared a *CDH23* reference sequence were identified in the four individuals. The SNPs were categorized to differentiate polymorphic from potentially pathogenic SNPs. This was done by first sorting the SNPs into exonic and intronic SNPs. Intronic SNPs are pathogenic only if they occur in a splice site or if they create a cryptic site. A variant in the splice site could prevent exons from being translated, which could result in termination of the protein. These pathogenic intronic SNPs occur most commonly at the start or the end of an intron, and were not the case for any of the intronic SNPs identified in this project

The intronic and exonic *CDH23* SNPs were classified as informative and non-informative, and the informative exonic SNPs were classified as synonymous and non-synonymous. Eight of the *CDH23* SNPs are exonic, informative and non-synonymous, and were considered potentially pathogenic. However, none of these SNPs co-segregated with deafness. These eight potentially pathogenic *CDH23* SNPs are listed in the paper by Astuto et al. (2002) as non-pathogenic, confirming that no mutation in *CDH23* is suspected to be pathogenic.

Twenty-one informative SNPs (12 exonic and 9 intronic) were used to construct the *CDH23* intrageneic haplotype. No *CDH23* intrageneic haplotype was shared by all three Family A members with hearing loss; all three had a different combination of paternal and maternal *CDH23* haplotypes. This finding is in agreement with the conclusion that no *CDH23* SNP is suspected to be pathogenic.

Possible Explanations for Genetic Cause of Deafness in Family A

Given the evidence supporting *CDH23* as a potential candidate gene, it is somewhat surprising that we did not identify a pathogenic mutation in *CDH23*. A number of possibilities exist which could explain the deafness in this sib-ship. Firstly as the deafness phenotype in sib-ship 2 differs in onset from other deaf Family A members, the deafness in this sib-ship may have a different genetic cause. A possibility is that the *PCDH15* mutation may not be contributing to the deafness in these family members, and is only present by coincidence. The deaf persons in sib-ship 2 could be homozygous for a mutation in a yet-to-be-identified gene. A genome-wide scan on this sib-ship would yield important data relative to this possibility; however using this sib-ship alone for a GWS may not be powerful enough to identify the causative gene.

A second possibility is that a gene residing within the deafness-associated haplotype, not yet classified as a deafness gene could be contributing to the deafness. However, a revision of all the genes within the deafness-associated haplotype region did not reveal any genes, functional or otherwise, which seemed to have deafness-causing potential. It is of note that although the genome has been mapped, not all genes have been

annotated (identified). Therefore, the disease-causing gene may reside within the deafness-associated haplotype, but cannot be identified because it has not been annotated.

A third possibility is the deafness in sib-ship 2 may have an environmental cause, such as the measles or mumps and the *PCDH15* mutation may not be contributing to the deafness. A review of the dates of outbreaks of hearing-related disease such as measles in Newfoundland would help address this possibility. It is the author's opinion that the deafness in this sib-ship may have an environmental cause, as the majority of the siblings are affected. The mutation in *PCDH15* may be coincidental, and these siblings may be phenocopies.

Conclusion

This study was conducted to determine if *CDH23* contributed to deafness in Family A. Sequencing the entire coding region of *CDH23* and all exon/intron boundaries in three deaf family members and a hearing family member did not identify any *CDH23* mutations. Initially, eight of the identified *CDH23* SNPs were suspected to be pathogenic because they were located in the coding region (exon) and were polymorphic. However they have since been classified as non-pathogenic because the SNPs did not segregate exclusively with deafness and all eight SNPs were documented by Astuto et al. (2002) as non-pathogenic.

Initially, *CDH23* seemed to be a promising candidate gene because of its proximity to the previously identified deafness-associated haplotype and due to its known interaction with *PCDH15*. However, the results from this study show that *CDH23* does not contribute to the deafness in Family A.

Chapter 3: Family B and Family 41

Introduction

Family History on Family B

Family B is a large deaf family, originating from a small community on the Southwest coast of Newfoundland. The complete pedigree documents 229 family members, spans seven generations and reports 14 cases of inbreeding. Twenty family members have been diagnosed with autosomal recessive, non-syndromic hearing loss. Figure 3.1 is a partial pedigree of Family B.

An extensive genealogy dating back to the family's founders has been collected by a present-day family member of Family B. The founder male was born in the early 1800s and was from England, and the founder female, also born in the early 1800s, originated from a small community on the Southwest coast of Newfoundland. They were wed in 1822 and had five children. The genealogy extends from its founders to its members in the present-day generation. It is a very complete pedigree, documenting most of the family names as well as extended family members who married into the family over the past 200 years. In all, more than 500 family members have been identified. The family history (names, birth dates, etc.) ascertained through this (independent) genealogy agrees with the data collected through the Newfoundland Medical Genetics Program, providing a level of verification of both data sets. The Newfoundland Medical Genetics Program has been collecting demographic information, birth measurements (weight, gestation), and birth defect diagnostic information from all live births occurring in the province since 1976. This information can be accessed for the purpose of research.

A study performed by Bear et al. (1987) compared the genetic relatedness of persons from Newfoundland communities at various distances. The findings reported that people from the same community and from communities in close proximity had a higher degree of genetic relatedness compared with people from communities which were more distant. Genetic relatedness was measured by using the average kinship coefficient (the probability that an allele selected at random will be identical in two individuals because it was inherited from a common ancestor). Most of the 500 family members in Family B are from one of three outports situated very close to each other along Newfoundland's Southwest coast. Therefore, it can be suspected that these family members, even those who married into the family, will have a high degree of relatedness.

Previous Research on Family B Identified *TMPRSS3* as Deafness-Causing Gene

Family history and DNA collection on Family B were performed by Dr. E. Ives (Newfoundland Medical Genetics Program). The information and the DNA samples were given to the Edward Wilcox laboratory in Rockville, MD, in an effort to identify the causal disease gene. A genome-wide scan was performed, which identified linkage to markers at the DFNB8/B10 locus in one branch of the pedigree (Ahmed et al. 2004). The deafness gene Tempress3 (*TMPRSS3)* resides at the locus of DFNB8/B10. The haplotype analysis of the linked markers at the DFNB8/B10 locus demonstrated homozygosity. Consequently, a deafness-associated haplotype was constructed. Family B members who carried at least one copy of the deafness-associated haplotype were sequenced in full for *TMPRSS3*. Two pathogenic alleles were identified by this sequencing. One of these pathogenic alleles, c.207delC in exon 4, is a known mutation, and has been described in

the Spanish, Greek and Pakistani populations (Ahmed et al. 2004). This mutation produces a frameshift in the reading frame of the translated DNA sequence, and is predicted to produce a protein which is missing three of the four conserved domains (Wattenhofer et al. 2002). The second identified *TMPRSS* mutation is a novel mutation, designated c.782+3delGAG, and occurs at the splice site between intron 8 and exon 9. This splice-site mutation is predicted to splice out upstream exons and may produce a prematurely terminated TMPRSS3 protein (Ahmed et al. 2004).

All Family B members who carried two copies of the *TMPRSS3* c.207delC mutation were deaf (persons 260, 246 and 249 in Figure 3.1). Two deaf siblings (291 and 285 in Figure 3.1) were heterozygous for the c.207delC mutation and also heterozygous for the novel c.782+3delGAG mutation. No hearing person was heterozygous for both mutations and no family member homozygous for the c.782+3delGAG mutation was identified. The two heterozygous mutations in *TMPRSS3* were identified as the cause of deafness in the deaf siblings. The genetic term for two single mutations in the same gene acting together to produce a disease phenotype is called compound heterozygosity; the two deaf siblings with the two *TMPRSS3* mutations are called compound heterozygotes. Recently, another case of compound heterozygosity in *TMPRSS3* has been identified as the cause of pre-lingual deafness in four German children (Elbracht et al. 2007). These findings on Family B were published by Ahmed et al. (2004). Although two *TMPRSS3* mutations were identified as causal for deafness, Ahmed et al. (2004) reported only a single mutation *TMPRSS3* haplotype and suggested this haplotype mutated twice.

However, it is more likely that two *TMPRSS3* haplotypes exist, each mutation segregating with its own haplotype. The error in the Ahmed et al. (2004) seems to be how

the *TMPRSS3* haplotype was constructed. A careful analysis by this author found that the data from the Ahmed et al. 2004 contained too few markers and the markers which had used were non-polymorphic genotypes (non-informative). Consequently, the resulting *TMPRSS3* haplotype was not characterized enough. In this project, additional markers and polymorphic *TMPRSS3* SNPs will be used to more fully map the *TMPRSS3* haplotype in selected family members to determine if two mutated *TMPRSS3* haplotypes exist in Family B.

Family History of Family 41

A mutation screen on the 48 ascertained deaf probands from the Newfoundland population was recently performed (Gamberg, personal communication). The probands were screened all known Newfoundland deafness mutations (one in *WFS1*, 2 in *TMPRSS3*,and one in *PCDH15)* and the two common connexin mutations (*GJB2* and *GJB6*). The proband in Family 41 was identified as a heterozygous carrier of the *TMPRSS3* c.782+3delGAG mutation, previously identified in Family B. Subsequently, the entire *TMPRSS3* gene (13 exons) was sequenced in the proband, his father and his mother (who was also heterozygous for the mutation but was hearing). No other *TMPRSS3* mutations were found (Gamberg, personal communication). Since the novel *TMPRSS3* c.782+3delGAG mutation is present in two unrelated families in a founder population, it was hypothesized that this mutation may be a Newfoundland founder mutation.

Goal for Family B

To determine if the two *TMPRSS3* mutations, c.207delC and c.782+3delGAG, segregates on separate mutation haplotypes.

Goal for Family B and Family 41

To determine if the c.782+3delGAG *TMPRSS3* mutation, identified in both Family B and Family 41, occurs on the same haplotype.

Objectives

1. Find informative (polymorphic) markers and *TMPRSS* SNPs within the boundaries of the previously defined *TMPRSS3* deafness-associated haplotype [as defined by Ahmed et al. (2004)]
2. Genotype the Family B compound heterozygotes and their immediate family members and construct the *TMPRSS3* haplotypes flanking each *TMPRSS3* mutation. Determine if the two *TMPRSS3* mutations occur on separate haplotypes.
3. Genotype the chromosomal area flanking *TMPRSS3* in the proband and mother in Family 41 using the informative markers and *TMPRSS3* SNPs and construct the haplotypes.
4. Compare the *TMPRSS3* c.782+3delGAG mutation haplotype in carriers in Family B and Family 41. If the haplotypes are identical, it is evidence this mutation is a founder mutation and the haplotype is a founder haplotype.

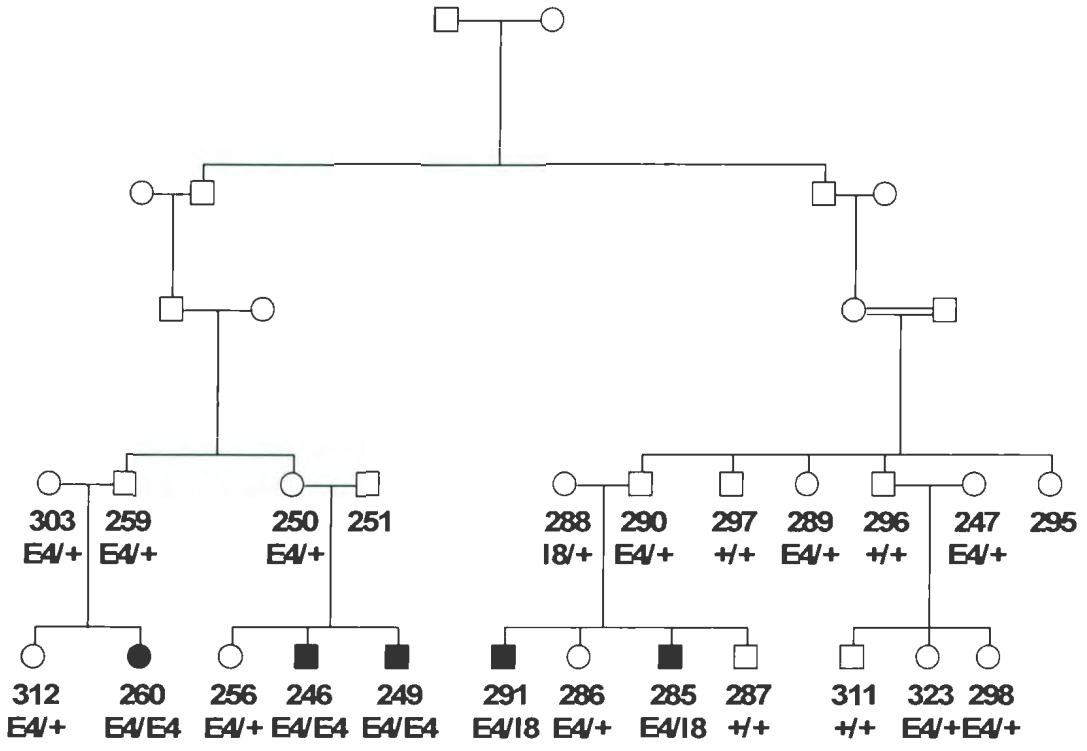


Figure 3.1 – Partial pedigree of Family B showing carrier status for *TMPRSS3* exon 4 mutation (E4) and the *TMPRSS3* intron 8 mutation (I8). The affecteds (black symbol) are diagnosed with severe-profound, non-syndromic, autosomal recessive hearing loss. Two sibs (291, 285) are compound heterozygotes for the *TMPRSS3* exon 4 mutation and the *TMPRSS3* intron 8 mutation. The + symbol indicates the person is wild-type for the mutation.

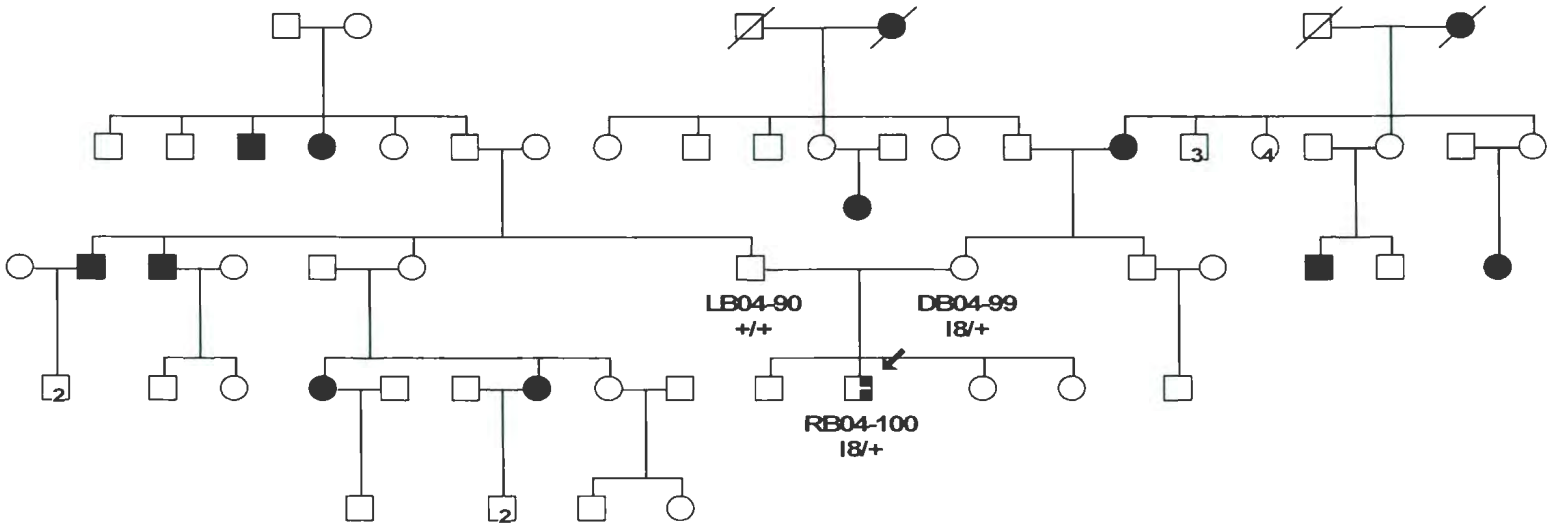


Figure 3.2 – Partial pedigree of Family 41 showing the identified carriers of the *TMPRSS3* intron 8 mutation (I8). The proband (RB04-100) and his unaffected mother (DB04-99) are heterozygous for the mutation (I8/+) and the proband's father (LB04-90) is wild type (+/+). DNA for other family members is not available. RB04-100 has a hearing loss in the right ear only (denoted by right side of symbol shaded).

Methods

Subject Recruitment

Family members for Family B were recruited through the Newfoundland Provincial Genetics Program, which was initiated in 1988 by Dr. E Ives. Family 41 was recruited through a province-wide program aimed at recruiting all Newfoundlanders with hereditary sensorineural hearing loss. Most Newfoundland health care professionals who provide specific services to the deaf and the hearing impaired are involved with this program. Research participants were required to give informed consent and donate a sample of blood. A signed consent form granted permission to access medical records and family history. Audiological tests were performed by a local physician to determine the type of hearing loss of affected individuals and to confirm normal hearing in non-affected persons. Physical exams (also performed by a local physician) were performed to determine whether affected persons were affected with other conditions (ie. syndromic hearing loss). Vision and vestibular functions were performed to test for Ushers syndrome. This project was approved by the Human Investigations Committee (HIC), Research Ethics Board of Memorial University, Newfoundland and Labrador (# 01.186).

DNA Preparation, PCR Thermo-cycling, and Electrophoresis

See Chapter 2, Methods, page 34

Protocol for Genotyping

A volume of 0.5µL of diluted PCR sample (1:10), 9µL of DMF and 0.4µL of Sequencing Size Standard was added to each reaction well in an optical plate. The

samples were denatured using a thermocycler, and were transferred immediately to the ABI 3130 for genotyping. Thermocycling conditions required for denaturing are found under Methods section in Chapter 2. The PCR products from the fluorescently labeled primers were detected by an ABI 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA) and genotyped using GeneMapper Software (ABI Prism, Version 3.5). The *TMPRSS3* haplotypes in Figure 3.3 and 3.4 were created manually and not by use of a computer program.

Markers and SNPs used to Construct the New *TMPRSS3* Haplotypes

The markers used to construct the *TMPRSS3* haplotypes were selected from Genome Browser (University California Santa Cruz, 2006). The marker heterozygosity was selected at ≥0.7 to maximize polymorphism. Marker heterozygosity is the chance a randomly selected person will be heterozygous for that marker. Table 3.2 is a list of microsatellite markers used to characterize the *TMPRSS3* exon 4 and intron 8 mutation haplotypes. The markers were selected based on location as well as heterozygosity; selected to be spaced ~ 1 Mb apart, starting from 10Mb proximal of the *TMPRSS3* gene and finishing at the end of the chromosome.

Table 3.3 is a list of the *TMPRSS3* SNPs used to characterize the two *TMPRSS3* mutation haplotypes. The *TMPRSS3* SNPs were identified through a different project which sequenced the entire *TMPRSS3* gene in all the Newfoundland deaf probands to identify any potentially pathogenic mutations (Gamberg, personal communication). The *TMPRSS3* SNPs selected to construct the *TMPRSS3* haplotype were highly polymorphic.

Family Members Selected for Genotyping

Five individuals from Family B were selected for *TMPRSS3* haplotyping, including the two deaf compound heterozygotes (291 and 285), their parents (288 and 290), and their unaffected sister (286) (Figure 3.1). Three individuals from Family 41 were haplotyped for *TMPRSS3* including the heterozygous proband (RB04-100), his unaffected mother (DB04-99, who was also heterozygous for the mutation) and his unaffected father (LB04-90) (Figure 3.2). Table 3.1 is a summary of the audiology reports and *TMPRSS3* mutation status from selected Family B members and the three Family 41 members.

Results

Figure 3.3 shows the results from haplotyping the area flanking the *TMPRSS3* exon 4 mutation in selected Family B members. The *TMPRSS3* exon 4 mutation haplotype originates from the father (290) and is inherited by the two compound heterozygotes (291 and 285) and the unaffected daughter (286). Their mother does not carry the exon 4 mutation and consequently does not share the *TMPRSS3* exon 4 mutation haplotype. It can be reasonably assumed the other *TMPRSS3* exon 4 mutation carriers in Family B share the same mutation haplotype.

Figure 3.3 also shows the results from haplotyping the *TMPRSS3* intron 8 in Family B. The data from the additional markers and *TMPRSS3* SNPs provides evidence that two mutated *TMPRSS3* haplotypes are present in Family B. As mentioned, the *TMPRSS3* exon 4 haplotype has been inherited from the father – an immediate family member in Family B. The two compound heterozygotes (291, 285) have inherited the *TMPRSS3* intron 8 mutation and mutated haplotype from the mother (288), who has married into Family B. No other Family B members have been identified as carrying the *TMPRSS3* intron 8 mutation (Figure 3.1). The *TMPRSS3* intron 8 mutation does not seem to originate from Family B; rather it is only present in one branch of the pedigree, brought into the family through marriage.

Figure 3.4 shows the *TMPRSS3* intron 8 haplotypes in Family 41. The deaf proband (RB04-100) has inherited the *TMPRSS3* intron 8 mutation (and *TMPRSS3* intron 8 mutation haplotype) from his unaffected mother (DB04-99). His father (LB04-90) is not a carrier.

The *TMPRSS3* intron 8 haplotype identified in Family B is identical to the *TMPRSS3* intron 8 haplotype in Family 41 (ie. the carriers share a span of alleles which flank the *TMPRSS3* intron 8 mutation). Table 3.4 outlines which alleles are shared among the carriers and the total span of DNA which is shared. Since family members in Family B and Family 41 share a common haplotype and a common disease mutation, the haplotype can be considered a founder haplotype and the mutation is a founder mutation. It is quite possible these families have a recent common ancestor.

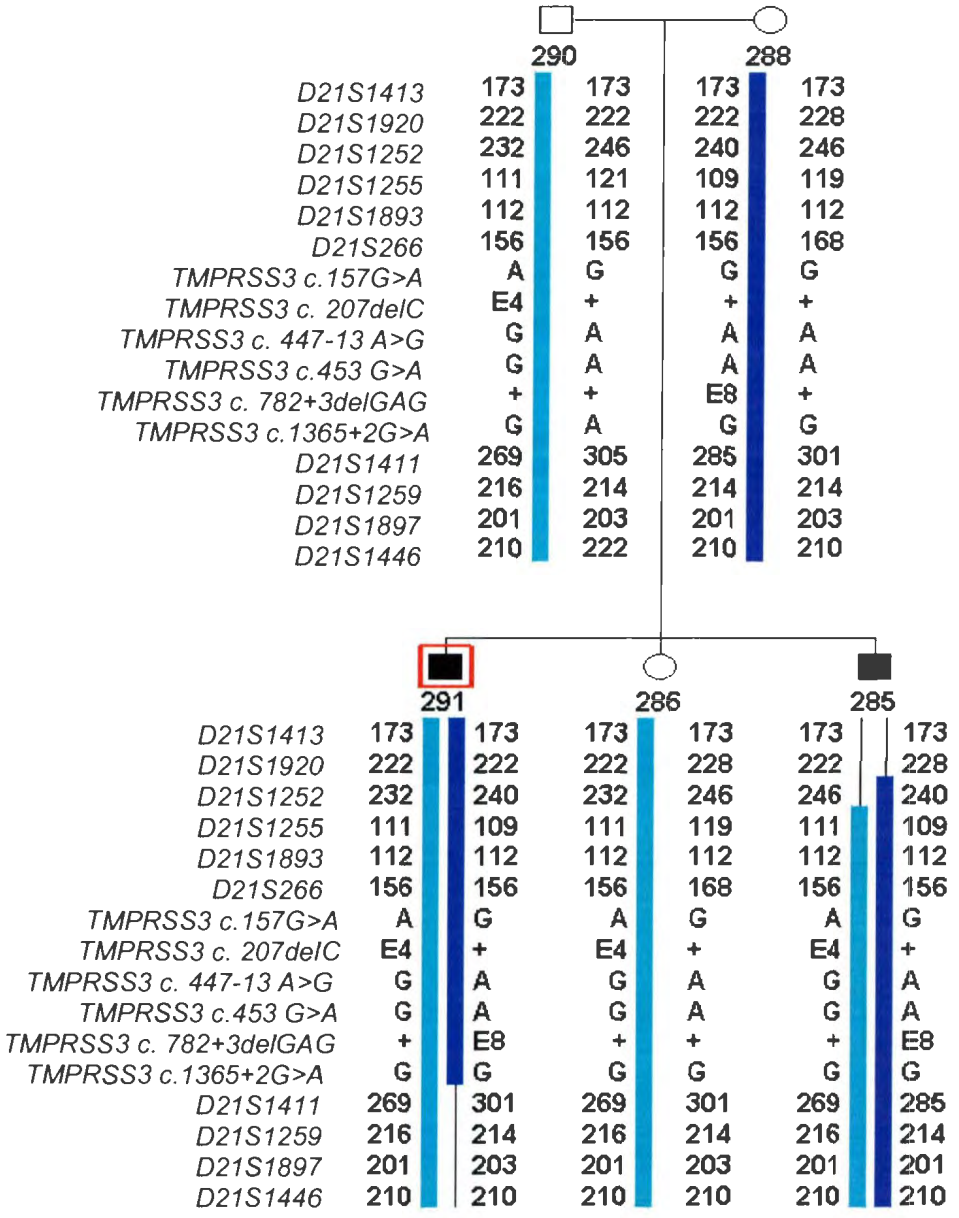


Figure 3.3 – *TMPRSS3* exon 4 and *TMPRSS3* intron 8 mutation haplotypes in two compound heterozygotes (291, 285), their unaffected sib (286) and two unaffected parents (290, 288) from Family B. The light blue bar is the *TMPRSS3* exon 4 haplotype, inherited from the paternal side (Family B) and carried by all three sibs. The dark blue bar is the *TMPRSS3* intron 8 haplotype, inherited from the maternal side (not direct member of Family B) and only present in the compound heterozygotes. Person 291 has a suspected cross-over on the *TMPRSS3* intron 8 haplotype at *D21S1411*. Person 285 has two recombinant haplotypes: the crossing-over occurs at *D21S1255* on the *TMPRSSS3* exon 4 haplotype and at *D21S1920* on the *TMPRSS3* intron 8 haplotype. All markers and SNPs are taken from Markers are taken from Genome Browser (University California Santa Cruz, 2006).

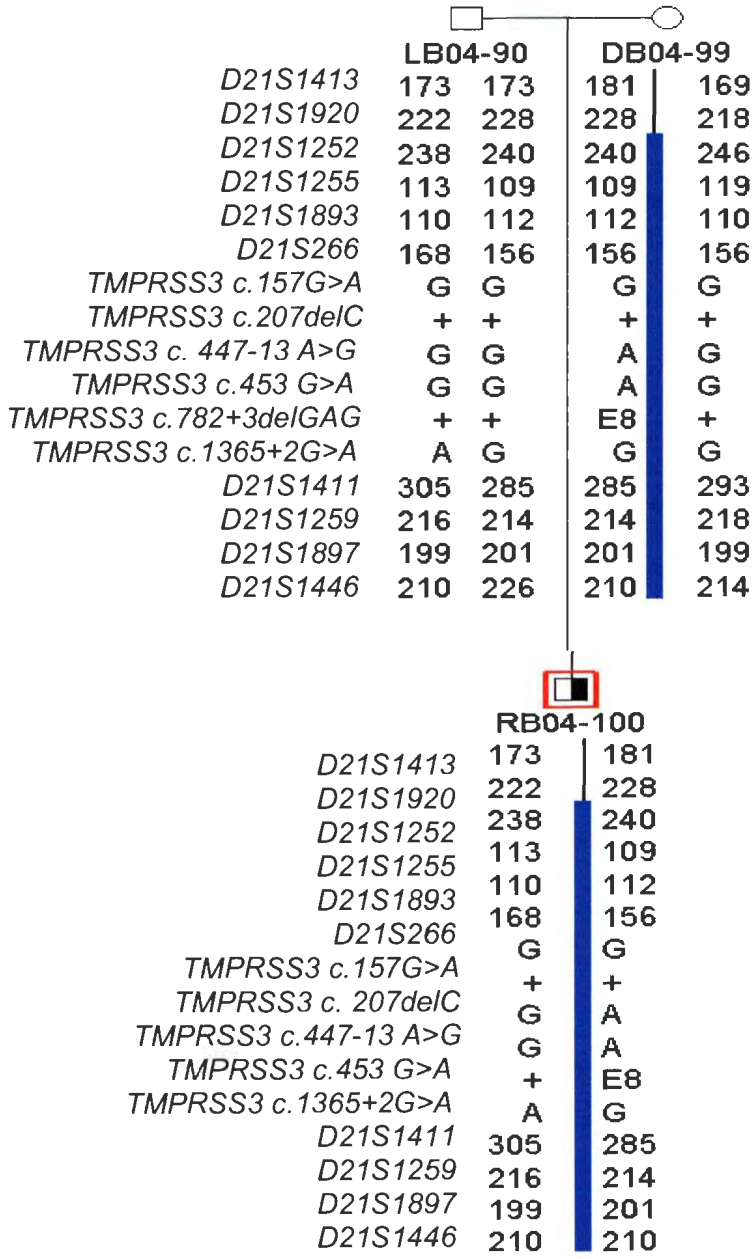
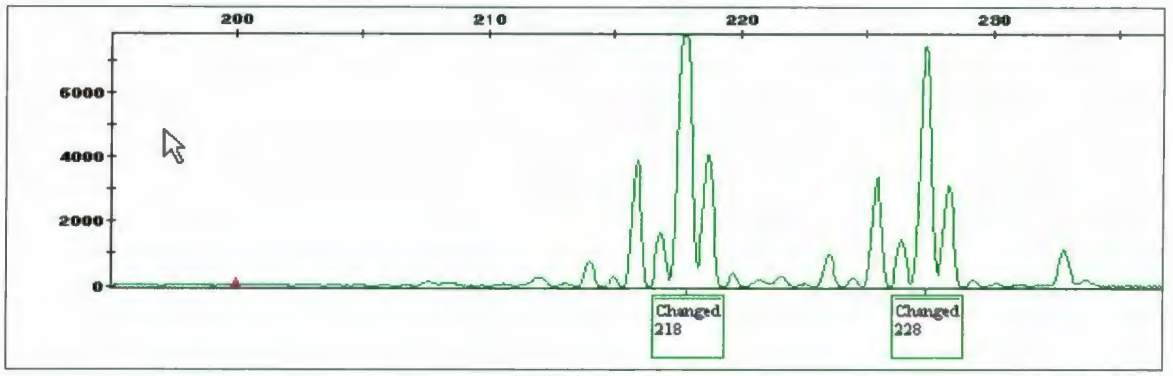


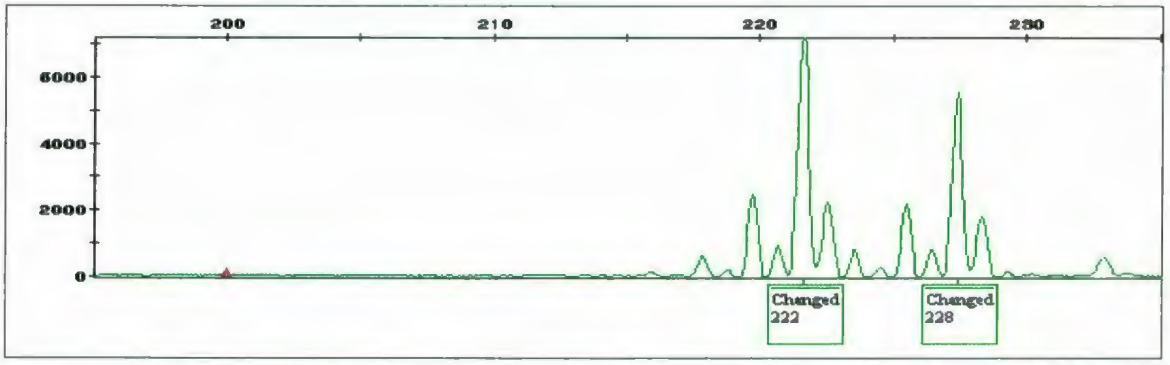
Figure 3.4 – *TMPRSS3* intron 8 haplotypes for affected proband (RB04-100) and unaffected mother (DB04-99) in Family 41. The father (LB04-90) is not a carrier of the *TMPRSS3* intron 8 mutation. The *TMPRSS3* intron 8 haplotype is highlighted in blue to show the common alleles with the carriers of the *TMPRSS3* intron 8 haplotype in Family B. Markers are taken from Genome Browser (University California Santa Cruz, 2006).

Location on 21q22.3 (bp)	Marker/ *TMPRSS3* SNP	Family B			Family 41	
		291	*288*	*285*	*DB04-99*	*RB04-100*
32 769 678	*D21S1413*	173	173	173	181	[181]
36 418 979	*D21S1920*	222	222	228	228	228
36 748 729	*D21S1252*	240	240	240	240	240
38 716 581	*D21S1255*	[109]	109	109	109	[109]
40 278 209	*D21S1893*	112	112	112	112	112
41 606 427	*D21S266*	156	156	156	156	156
42 665 226	*TMPRSS3 157G>A*	G	G	G	[G]	G
42 665 376	*TMPRSS3 c.207delC (E4)*	wt	wt	wt	wt	wt
42 665 503	*TMPRSS3 c.447-13A>G*	A	[A]	[A]	A	A
42 665 522	*TMPRSS3 c.453G>A*	A	[A]	[A]	A	A
42 665 854	*TMPRSS3 c.782+3delGAG (I8)*	I8	I8	I8	I8	I8
42 666 435	*TMPRSS3 c.1365+2G>A*	G	G	G	G	G
43 033 713	*D21S1411*	301	285	285	285	285
44 148 293	*D21S1259*	214	214	214	214	214
45 369 052	*D21S1897*	203	201	201	201	201
46 862 011	*D21S1446*	210	210	210	210	210

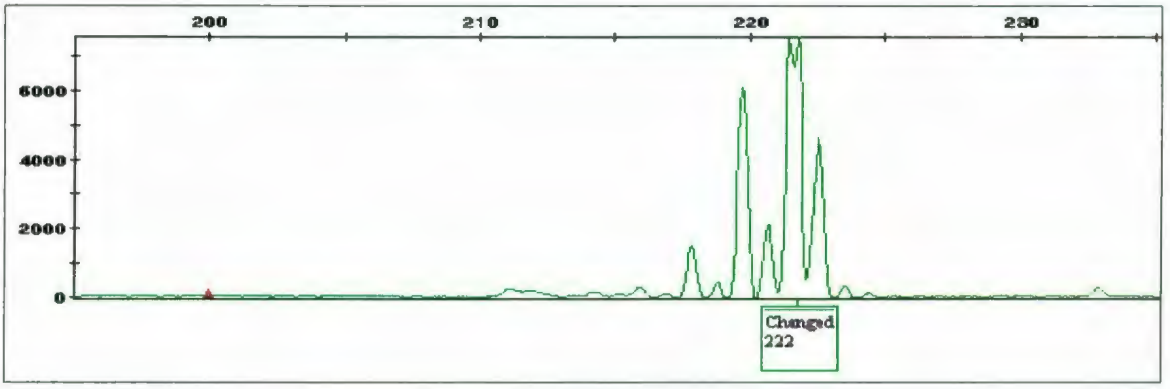
Figure 3.5: *TMPRSS3* intron 8 Haplotype Identified in Family B and Family 41 Members. Family B members 288 and 285 share a span of ~10.12 Mb with Family 41 members DB04-99 and RB04-100. This shared haplotype includes 8 markers, 5 *TMPRSS3* SNPs and one *TMPRSS3* mutation (intron 8) and is located on chromosome 21q22.3. The green region highlights the shared alleles.



DB04-99



RB04-100



290

Figure 3.6 – Examples of Genotypes for Marker *D21S1920* (GeneMapper). Homozygosity or heterozygosity of a marker is determined by the number of times a band pattern is present (ie. the pattern of vertical peaks, in this case green). If the band pattern is present only once, the person is a homozygote (ie. 290); if the band pattern is present two times, the person is heterozygous (ie. DB04-99).The numbers in the boxes under the band pattern are the genotypes, or alleles. For example, 290 is homozygous (222,222) and RB04-100 is heterozygous (222,228) for marker *D21S1920*. .

Table 3.1: Summary of Audiology Reports from Selected Members from Family B and Family 41

Ped #	Family	Gender	Type of Hearing Loss	*TMPRSS3* Mutation
246	B	M	profound, bilateral sensorineural hearing loss	E4/E4
247	B	F	none	E4/+
249	B	M	profound, bilateral sensorineural hearing loss	E4/E4
250	B	F	none	E4/+
251	B	M	none	E4/+
256	B	F	none	E4/+
259	B	M	none	E4/+
260	B	F	profound, bilateral sensorineural hearing loss	E4/E4
285	B	M	profound, bilateral sensorineural hearing loss	E4/I8
286	B	F	none	E4/+
287	B	M	none	"+/+"
288	B	F	none	I8/+
289	B	F	none	E4/+
290	B	M	none	E4/+
291	B	M	profound, bilateral sensorineural hearing loss	E4/I8
295	B	F	none	-
296	B	M	mild/moderate/severe	"+/+"
297	B	M	none	"+/+"
298	B	F	none	E4/+
303	B	F	moderate in RE	E4/+
311	B	M	none	"+/+"
312	B	F	none	E4/+
323	B	F	none	E4/+
DB04-99	41	F	none	I8/+
LB04-90	41	M	none	"+/+"
RB04-100	41	M	mod/severe in RE	I8/+

Table 3.2: Microsatellite Markers used to Build the *TMPRSS3* Haplotypes Genome Browser (University California Santa Cruz, 2006)

Marker	Location On 21q22.3 Mapped from the Centromere (bp)	Microsatellite Repeat Type	Heterogeneity (>0.7)	Product Size (bp)
D21S1413	32 769 678	Tetrenucleotide	0.875	160-200
D21S1920	36 418 979	Dinucleotide	0.740	220-234
D21S1252	36 748 729	Dinucleotide	0.804	231-251
D21S1255	38 716 581	Dinucleotide	0.803	100-140
D21S1893	40 278 209	Dinucleotide	0.760	100-130
D21S266	41 606 427	Dinucleotide	0.604	140-180
D21S1411	43 033 713	Tetranucleotide	0.933	220-250
D21S1259	44 148 293	Dinucleotide	0.670	200-240
D21S1897	45 369 052	Dinucleotide	0.740	188-214
D21S1446	46 862 011	Tetrenucleotide	n/a	200-240

Table 3.3: TMPRSS3 SNPs used to Create the *TMPRSS3* Exon 4 and Intron 8 Haplotypes

***TMPRSS3* Sequencing SNPs**	**Exon/Intron**	**Reference**	**Pathogenic**
TMPRSS3 157G>A	Exon 3	Scott, 2001	no
TMPRSS3 207delC	Exon4	Wattenhofer, 2002	yes
TMPRSS3 447-13A>G	Intron 5	Wattenhofer, 2002	no
TMPRSS3 453G>A	Exon6	Scott, 2001	no
TMPRSS3 782+3delGAG	Intron 8	Ahmed, 2004	yes
TMPRSS3 1365+2G>A	Exon13	Scott, 2001	no

Discussion

Family B is a large, consanguineous family from the Southwest Coast of Newfoundland. Twenty family members have been diagnosed with non-syndromic, autosomal hearing loss. In 2004, work performed by the Wilcox lab determined the cause of deafness in Family B was due to two mutations in *TMPRSS3:* c.207delC in exon 4, a known mutation, and c.782+3delGAG in intron 8, a novel mutation (Ahmed et al. 2004). Most of the deaf family members were homozygous for the c.207delC mutation, but two deaf siblings (291 and 285, Figure 3.1) were heterozygous for the c.207delC mutation and the c.782+3delGAG mutation. The cause of their deafness was explained by the interaction of these two single mutations in *TMPRSS3,* called compound heterozygosity.

The paper published on this work (Ahmed et al. 2004) reported the two *TMPRSS3* mutations had occurred on the one haplotype and that the "founder haplotype" had mutated twice. This is an unlikely scenario however, as the DNA mutation rate (in the human species) is generally low (Strachan and Read 1999). It was hypothesized only one mutation haplotype was found was because not enough informative markers were used. One of the goals of this current project was to more create a more complete *TMPRSS3* haplotype using more informative markers and *TMPRSS3* SNPs to determine if a second mutated haplotype exists in Family B.

The results from haplotyping the two *TMPRSS3* mutations with the additional markers and *TMPRSS3* SNPs in selected Family B members provided evidence that two distinct *TMPRSS3* mutation haplotypes exists, and that each mutation segregates with its own haplotype (Figure 3.3). It is suspected that Ahmed et al. (2004) did not use enough informative markers/SNPs so that the second haplotype was missed.

During the process of a mutation screen of all the deaf Newfoundland probands, the proband from Family 41 and his unaffected mother were found to carry a single copy of the *TMPRSS3* c.782+3delGAG intron 8 mutation, the same mutation identified in the compound heterozygotes in Family B. Both parents in Family 41 originated from the Burin Peninsula (NL). It was suspected that this mutation may be a founder mutation, and that the c.782+3delGAG *TMPRSS3* mutation was passed on to both families from a recent common ancestor. The second goal in this study was to determine if the *TMPRSS3* c.782+3delGAG is a founder mutation. This was determined by comparing the *TMPRSS3* intron 8 mutation haplotypes (c.782+delGAG mutation) in carriers in Family 41 and Family B to determine if the haplotype is identical.

The *TMPRSS3* intron 8 mutations haplotypes in mutation carriers in Family B and Family 41 are identical. As shown in Figure 3.5, two Family B members (291, 285) and two Family 41 members (RB04-100, DB04-99) share a span of 10.1 Mb of DNA, which includes 8 informative markers, 5 informative *TMPRSS3* SNPs, and the *TMPRSS3* intron 8 mutation. This is sufficient evidence to conclude that the haplotype is a founder haplotype, the intron 8 mutation is a founder mutation, and that the two families are related through a recent, common ancestor.

It is possible that this common ancestor may be one of Newfoundland's first settlers. If this is true it would be expected that there would be a high carrier rate of the c.782+3delGAG founder mutation in the Newfoundland population.

To date, it is not known how Family B and Family 41 are related. Family B is from the Southwest coast, and Family 41 is from the Burin Peninsula. It is important to recognize that the origin of the *TMPRSS3* intron 8 mutation in Family B is the mother

(288) who has married into the family (the only other Family B members who have the mutation are her children, 291 and 285). Therefore, in order to identify a common ancestor of the mutation carriers in Family B and Family 41, it is important that the family of the carrier mother (288) in Family B be researched. To date, the only family history available on the carrier mother is that she originates from the same community as Family B.

Future Directions for Research on Family B and Family 41

Further work is needed to fully solve the genetic deafness in Family 41. To date, the only mutation identified in the family is the *TMPRSS3* intron 8; as the deafness segregates in an autosomal recessive pattern, the deaf proband must harbour a second mutation. He has already been screened for all Newfoundland deafness mutations and sequenced the remaining *TMPRSS3* exons but a second mutation has not been found (Gamberg, personal communication). The only DNA available for Family 41 is on the proband and his parents. The pedigree for Family 41 shows that both parents have deaf relatives. If the DNA from these relatives could be collected, linkage analysis could be performed which might identify the second gene which explains the deafness in the probands and identify a novel deafness gene. However, it must be noted that his hearing loss occurs in only one ear, which is an atypical phenotype compared with others in his family and it is possible that he is a phenocopy. If this is the case, the results from linkage analysis would be skewed.

Although not discussed in this thesis, Family B has a number of deaf family members who could not be fully explained. The majority unexplained are affecteds who are heterozygous for the *TMPRSS3* exon 4 mutation; as well, number are wild-type for both *TMPRSS3* mutations. A follow-up for Family B could be to re-examine the results from the previously genome-wide scan to determine if there is a second significant LOD score which could point to the locus of a second mutation.

Conclusion

Previously, Family B was studied to determine the genetic cause of deafness. Two mutations in the deafness gene *TMPRSS3* were identified as causal but only one mutation haplotype was defined. The current project used additional informative markers and polymorphic *TMPRSS3* SNPs to generate more informative *TMPRSS3* haplotypes. With the additional data, it was found that two distinct *TMPRSS3* mutation haplotypes exist in Family B which segregate independently with the two mutations. These *TMPRSS3* mutation haplotypes can be used in the future to compare with other identified *TMPRSS3* mutation carriers in the Newfoundland population.

Subsequently, a deaf individual from Family 41 and his unaffected mother were identified as heterozygotes for the *TMPRSS3* intron 8 mutation, the same mutation as identified in Family B. The *TMPRSS3* intron 8 mutation was haplotyped in the carriers in Family 41 using the same markers and *TMPRSS3* SNPs as used in Family B. It was found that two mutation carriers of the *TMRPSS3* mutation in Family B and two mutation carriers in Family 41 share a common *TMPRSS3* intron 8 mutation haplotype which spans 10.1 Mb and includes 8 markers, 5 SNPs and the intron 8 mutation. This suggests that the *TMPRSS3* intron 8 mutation is a founder mutation and its' haplotype is a founder haplotype. Furthermore, it is quite possible that Family B and Family 41 are related through a common ancestor. Further work is needed to determine how the two families are related.

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Appendices

Appendix A: How to Genotype using Radioactive Samples. How to Read a Genotyping Gel

DNA was extracted from whole blood using common procedure. Using the forward and reverse primers and a cocktail of appropriate buffers and enzymes, the DNA region of interest was isolated and amplified on an automated thermocycler (see Methods section, Chapter 1). A radioactive nucleotide (dGTP) was added to the dNTP mix in place of an inert dGTP, as a means of radioactive labeling. The amplified, radioactive PCR product was run on a 6% acyrilamide gel (slab gel). The time needed to run the samples on the gel depended on the base pair fragment length of the PCR products (smaller base pair fragment, shorter length of time).

A sheet of white corrugated paper was placed on the finished gel to transfer the radioactive products, and then peeled away from the gel. The corrugated paper was then placed in a cassette containing a film negative for a few days, exposing the radioactive samples to the film. The PCR products could then be visualized using a light box with the film negative.

The PCR products on the film negative appear as staggered bands running down vertically, which are differentially shaded. The various shading corresponds to different alleles. The darkest bands are the true alleles, while the lighter bands are "shadow bands" – bands which migrate with the true alleles. Each marker amplifies a particular pattern of alleles, consisting of dark bands (true alleles) and shadow bands. A homozygote will have one clear pattern of bands. A heterozygote will have two copies of the band pattern which may be spaced apart from each other, making the two copies very

obvious, or they may overlap, so that the patterns are hard to distinguish. Related persons will share the same pattern of bands; for example a child will have a band pattern inherited from his mother and another inherited from his father.

The highest dark band (also the highest molecular weight) is numbered 1, and the bands further down are numbered sequentially. This process is termed genotyping.

Appendix B: Deafness genes identified in the Newfoundland population (Gamberg, personal communication)

	Mutation Name	Amino acid change	# NL Families	Lit Reference
GJB2/CNX26	35delG	Truncation	7	Denoyelle, 1997
GJB6/CNX30	ΔD13S1830	Truncation	3	del Castillo, 2002
TMPRSS3 DFNB8/10	207delC	Truncation	1	Ahmed, 2004
TMPRSS3 DFNB8/10	782+3delGAG	Splice site	2	Ahmed, 2004
PCDH15/DFNB	1978T>A	V528D	1	Ahmed, 2003
WFS1/DFNA	2146G>A	A716T	1	Young, 2001

Appendix C: Primer Sequences, Expected PCR Product Size and PCR Conditions Used for *CDH23* Primers. Taken from Bolz et al. 2001. Primers used the following Thermo-cycling Protocol: 95°C for 3 min; three cycles at 95°C for 15 s, AT+4°C for 10 s, 72°C for 30 s; three cycles at 95°C for 15 s, AT+2°C for 10 s, 72°C for 30 s; 30 cycles at 95°C for 15 s, AT for 10 s, 72°C for 30 s and 72°C for 10 min.

Amplified Exon	Primer Name	Primer Sequence	Size of Fragment (bp)	Annealing Temperature
1	1f 1r	GCTATACCCAGGATAGGACAATGT CCTGTGAGAGCTGCAGAAGGCAAC	465	53
2	2f 2r	CCTGTGTCACCTTATAGAGTGTGT GATGACCTCAACCTGTAAGATCCC	242	55
3	3f 3r	GGAAGGCATAAACGTGACCTCTC GATGCCACTGTGGAGTCAGAATGG	327	55
4	4f 4r	AAGCTCCTAGGGCAATCCTGGAGC CACCTTTCCGTGTGATCACCTGG	291	50
5	5f 5r	GGGTCACAGGATTTCTGGACCC TTCACCTACACCATGGTGGTCTGG	248	55
6	6f 6r	GGCTGAAGGATGTAGAGAATGG TGTGTGCCACTGGGTCAATGTCC	396	55
7	7f 7r	CCTTCCCTGCTGGAGTGCAAGAGCA GCTGTGCCAGAACACTCATCACTGC	441	55
8	8f 8r	GCTGTGGGTGCCATGATAGCTA CCTCAGAGCCTGAGATGCCTACT	241	55
9	9f 9r	TGAGTCTTTAATGCCCAGAGAGG CCTCGGACACTGCTGGAGGTTG	307	55
10	10f 10r	GGTAAGCAAGAGCTGTTGCTGTG TTATCCTCAGAACCTACCAGGAG	391	55
11	11f 11r	CCAGAAGCTATGGCCCATCAGAGG TGTGACAGTTTGAACAGGTGACACC	373	55
12	12f 12r	CCCAACCAAAGCAGCTCTGGG GGATCTAAGGGAATCTTCTACC	351	55
13	13f 13r	CAGAGACTCTAACAGGTGCTCTGG CCTGATTGCTGAGGTCCCTTGGAC	377	55
14	14f 14r	CCAAAGGAGACGTGCGAGAGGAAC GTTCCCAGATCTCATGAGTCTGG	236	55
15	15f 15r	GGCAAGGACAGGCTGGGAAATGC CAAGCAAGCAAGTACAGGGAGAAG	394	55
16	16f 16r	GCCTCCAGTTGAAGCACAAGGG TCCTGAGTAGCCCAGAGTGTCAGG	414	55
17	17f 17r	CCATGCCAGCCATAACTTCTCTGC GTTCACAGGAAGCACTCACCATCC	292	55
18	18f 18r	CAGACCTAGCCTGACTCCTTGGTG CCTCCCACAATTTGTGTGCAGAGC	276	53

Appendix C: Primer Sequences, Expected PCR Product Size and PCR Conditions Used for *CDH23* Primers

Amplified Exon	Primer Name	Primer Sequence	Size of Fragment (bp)	Annealing Temperature
19	19f 19r	CACCTCCCTGAGAGCTGGTCACTG CTGGTGCTCAAAGCTGGTCTGCTC	390	55
20	20f 20r	CCAGATCATGGTAGCTTGCTAAC CACAAAGCCTCACACTGGCTCAG	355	55
21	21f 21r	GGTGGAGCTGGCAGAATTAATGC CTATTGCAAGAGCCAGCTCAGAG	366	55
22	22f 22r	ATCTTAGCTGGAATACAGACTCCC GGTGATCAGACATAGACCTCCTG	535	55
23	23f 23r	CACCACTTGCCTTCTTCCTGTCC CTCTGTCCTTCAAGACCCAGCTC	352	55
24	24f 24r	CTAGCTGCAGCCAAGTGTGGCTTG CCTACAAGTCCAATAGACAGCAG	443	55
25	25f 25r	CTACTTGGTCTGGTGCTGGAGAC CACCTAGTCTGGCTGTCCATCTG	327	55
26	26f 26r	CGCATCAGAGCACCTCAGGCAG GTAATGTTTGGTGAGTGCCTGGTGG	412	55
27	27f 27r	GGAGTGTGCAAAGTCACAGGAAG CCTCTGGTGCCACTGAGCATGTG	316	55
28	28f 28r	CGTGAAGGGAAGGAAAGGAACTC GACCTTTGGCAGCCTAGAGAAGC	227	55
29	29f 29r	CCAACCATGGCAGGCCAGCACAG CAACTAATGGCCTTCTCTGTTC	304	55
30	30f 30r	CAGGAAAGCAGTGACCACACAAG CAGGGATGACCGTTTCGAAGGAG	328	55
31	31f 31r	GTGGCAGCTTGAGAAGCCACAGC GTGCACACAGAAGGAGCTCAACC	538	53
32	32f 32r	GGACAGAGGAAGTGACATGGAGG CGAAGCCTCAGGCTATCAAGACC	335	53
33	33f 33r	GCTAGGATGAGACCTCAGGCAGGT ACTGGCCACAGCCAACAGCACAG	196	55
34	34f 34r	GTTTGCTGATGTTCCAGAACCCAC GAAGAACCTGGTGCTTGGTGATG	382	53
35	35f 35r	CCAAAAGCCCTTGCTCAACAGAG CTCCTGGATGGAGAGTAGTGACC	327	53
36	36f 36r	GATTGCACAGCCCTTTTGGACTC GTTCCCATCCATGAGATCTGAGG	338	55
37	37f 37r	CTACAGGAGCAGGTGCCAGACTG CCTGCTGTGTGGAATTCCATTCTC	404	53
38	38f 38r	GAGTCACATGGAGTGAGTTCAGC CTAGACTGCATCTTTGCTCCATCC	425	55

Appendix C: Primer Sequences, Expected PCR Product Size and PCR Conditions Used for *CDH23* Primers.

Amplified Exon	Primer Name	Primer Sequence	Size of Fragment (bp)	Annealing Temperature
39	39f 39r	CCATCACAGCTCCAATGTCAGG GTGAGTTGCTGAGATTAGGACAGG	371	55
40	40f 40r	GATAACTCATCCACATGCATAGC CACACAATGGTGAGGCCAAATGC	460	53
41	41f 41r	GAGGCTTGCTAGAGGAAGCAGAAG CTCTTGGCACAAGGGTAACAATG	318	55
42	42f 42r	GAACCTCCTCCTCGGTTGCCATGC GTGAATCCAAGATTCCATGCTTCC	448	55
43	43f 43r	CCAAGCAGAGCAGTTGGCATCTG CAGTGTGTGTCCAGGTATCAGTGG	554	55
44	44f 44r	CACTTTGCTGAGCATGGCCTACAAC CTGTCTCTTCCAACCAGACCTGTGG	367	53
45	45f 45r	CCAGGCATCTGCGTTCAGTCTGC CCAGAATCAGGCTGGGAGTGCTGG	353	55
46	46f 46r	GCAAGAGTCTTGAAGACAGCAGAG GTGTCCAACCTGAGGCTCCAGTCC	535	53
47	47f 47r	GGGACTTGAGAAGATCACCAACAA GCTGCTCAAGGGAGGATGAGGAAG	764	55
48	48f 48r	CGTGACCCAAGCTTATGAGGAGG CCTGATTGGCTCTGAGTGACCAAT	339	50
49	49f 49r	CAAGTGAGCACACCACTGGAAAGC CTGGAATCTGGCATGGAATGCTGG	550	53
50	50f 50r	CTCCTCATACTTTGGAGAGCTGC TGCAGGCATCAAGCTGCTCGTGG	375	53
51	51f 51r	ATGCCTGCATCAGTGAGTTCTAGG TGGTGGCTGCTCAGTCTCTGACC	294	53
52	52f 52r	CTGGTCAGGCAGGTAGCTCCAGG CTTGCTAGAGGCTTTGCTTGTTGC	423	53
53	53f 53r	AGGCTTACTCCTGCATGACCAGG CCAAGACATCATGGGTGTCCAAG	295	53
54	54f 54r	CTTGGACACCCATGATGTCTTGG CCACAGCTGGAAGAAGGAAATACT	387	53
55	55f 55r	GCTGTTGAGGACATTCTGCTACG GGAAGAGCACAGTCAGAGGAAA	465	55
56	56f 56r	GCTACAGCTTCCTGAAGACTGCG TGTCTAGCCAAATGTGTATTCTGGC	511	53

Appendix C: Primer Sequences, Expected PCR Product Size and PCR Conditions Used for *CDH23* Primers

Amplified Exon	Primer Name	Primer Sequence	Size of Fragment (bp)	Annealing Temperature
57	57f 57r	CCTCAGCACCTACAAAGTCACTGC GGCATATGTGGGTCATCTCTAGC	291	53
58	58f 58r	GCTAGAGATGACCCACATATGCC CCAGCACCTGGATCAACTCTGAGC	449	55
59	59f 59r	AGTACACTGCAGGTGCAGGGACTG CATGCAGTGACTGAGAGGTCATGG	308	53
60	60f 60r	CCACTTGCCTGTCACCTTTGCTC CCAATCACTTCATCCACACTTGG	654	57
61	61f 61r	CCAAGTGTGGATGAAGTGATTGG GAATACTCCCATGTTACAGATGG	500	55
62+63	62+63f 62+63r	GGTTGTTGAACTTTGGAGGCTTGAG TACCAGCTGAGCCAGCCACAATGG	541	53
64	64f 64r	CGATCATCGTCCTGGCTATCCTCC CCTGAGCCAGGCTTGTGGCTCC	327	55
65	65f 65r	GGACACAGGTGAGAAGGCAGTGG CCTGTCAGCCACGTGGTTTCTGAGG	421	55
66	66f 66r	CCAAGTCAGTGAAAGGCACTATATG CCAGAGGTTGCAAAGACCCAGC	318	55
67	67f 67r	CCAAGAAGCTGGGTTCTTTAAGG CCACCCTGTGTTCTCTGGAGTGG	288	55
68	68f 68r	CCTAGGATCACCAGGATCAGATGTC TCCTGGCTGCAGAGCTCCAGAGA	330	55
69	69f 69r	CCAAGGTTCAGCCACATAGCCAGT AAGTGGGATCTGAGCAGCTGATGC	589	57