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dissertation entitled

Mapping A New Autosomal Dominant
Hearing Loss Locus, DFNA20, On 17Q25 And Searching
For The Disease Causing Gene(s)

presented by

Sainan Wei

has been accepted towards fulfillment
of the requirements for

Ph.D. degree in 2002

Major professor

Date 11.4.02

MAPPING A NEW AUTOSOMAL DOMINANT HEARING LOSS LOCUS, DFNA20, ON 17Q25 AND SEARCHING FOR THE DISEASE CAUSING GENE(S)

BY

Sainan Wei

A DISSERTATION

Submitted to
Michigan State University
In partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Graduate Program in Genetics

2002

ABSTRACT

MAPPING A NEW AUTOSOMAL DOMINANT HEARING LOSS LOCUS, DFNA20, ON 17Q25 AND SEARCHING FOR THE DISEASE CAUSING GENE(S)

By

Sainan Wei

MSUDF1 family is a mid-Michigan family of English descent with a form of nonsyndromic, genetic, late-onset, bilateral, progressive, sensorineural hearing loss. This locus was designated DFNA20 locus by the nomenclature committee of the Human Genome Organization in 1999 and contains a gene that is responsible for a hearing loss of dominant inheritance. It is the 20th locus discovered for the autosomal dominant hearing loss.

The gene for the hearing loss in this MSUDF1 family was mapped using a strategy of whole genome screening after the exclusion of known hearing loss loci. Positive linkage was found to the microsatellite marker, D17S784, on 17q25 near the telomere of the long arm (q) of chromosome 17. Haplotype analysis using additional markers on 17q25 defines the obligate region to an approximately 5.5 cM.

Ten possible candidate genes in this critical region were analyzed, these are CNCNG, GRIN2C, FKHL13, ACTG1, SPARC, ERBA2L, ARHGDAI, P4HB, DNEL1 and GALR2. CNCNG, GRIN2C, FKHL13, ACTG1 are clearly fine-mapped outside of the DFNA20 locus using two RH map panels, G3 and TNG4 from Stanford. SPARC and ERBA2L are spurious or incorrectly assigned on 17q25. The rest of the candidates,

ARHGDAI, P4HB, DNEL1, GALR2 were sequenced including the intron/exon boundary. These genes contain no mutations and were ruled out as the causative gene(s).

As the sequenced data from HGP and Celera were obtained, new microsatellite markers and SNPs were identified and used to narrow the DFNA20 interval to a region of about 1.5 megabases and a partial physical contig of approximately 1 megabases was also constructed.

This interval contained no known genes but has many novel partial transcripts. To select potentially important genes, ear expressed transcripts were identified with the help of a human cochlear cDNA library made at NIDCD and a mouse cochlear gene expression database constructed at the Corey lab at Harvard Medical School. Four interesting transcripts were found. These transcripts were characterized by multiple tissue northern blot in order to look for the tissue expression profile. Full-length transcripts were compiled by sequencing I. M. A. G. E clones and using EST data from HGP. The four transcripts were then sequenced on affected chromosome first. Any suspect changes were rechecked on the somatic hybrid carrying the normal chromosome. No significant changes were found. These four ear expressed transcripts are also excluded from consideration as causative gene(s) in this MSUDF1 family.

This dissertation is dedicated to:

My beloved grandmother, Lizhi Wang, whose love in my childhood always encouraged me on the way to my career; my uncle; Yaqing Wei, whose care for my education kept me on a good track towards my career; and my parents, Chuqing Wei and Zhuying Wang for their love, understanding and support.

ACKNOWLEDGEMENTS

I am most grateful to Dr. Rachel Fisher and Dr. Karen Friderici for their excellent guidance, patience and encouragement. I am also very grateful to my other committee members: Dr. Jill Elfenbein and Dr. John Fyfe for their support, encouragement, suggestions and enthusiasm. I would like to thank Dr. Tom Friedman, Dr. Rob Morell and other scientists in NIDCD of NIH for their enthusiastic initiative, help and guidance.

I would like to greatly appreciate Kathy Nummy, Mei Zhu, and Rebecca Bedilu for their expert advice and critical guidance of my work and interesting discussion. I also appreciate Rebecca Lucas, Christopher Vlangos for their kind advice regarding to both my lab work and my writing. I would like to extend my thanks to the whole wonderful research group in Dr. Karen Friderici's lab.

Last, I would like to thank my husband for his critical encouragement during the journey to my degree, which I never really cherished before. I thank my lovely daughter for making my life colorful and making me continue to learn new things.

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SYMBOLS AND ABBREVIATIONS

A	adenosine
aa	amino acid
BAC	Bacterial Artificial Chromosome
C	cytidine
cDNA	complentary DNA
cR	centiRad
DFN	Deafness, X-linked loci
DFNA	Deafness autosomal dominant loci
DFNB	Deafness autosomal recessive loci
ERM	ezrin/radixin/moesin family
EST	Expressed Sequence Tag
G	guanosine
GSP	Gene specific primer
GSS	Genome Survey Sequence
HL	Hearing Loss
HTGS	High Throughput Genome Sequence
I.M.A.G.E.	Integrated Molecular Analysis of Genomes and their Expression
LOD	Logrithm of Odds Ratio
NIDCD	The National Institute on Deafness and Other Communication Disease
NIH	National Institute of Health
NSHI	Nonsyndromic Hearing Impairment
nt	nucleotide
PCR	Polymerase Chain Reaction
RACE	rapid amplification of cDNA ends
RFLP	Restriction Fragment Length Polymorphism
RH	Radiation Hybrid
RT-PCR	reverse-transcription of polymerase chain reaction
SNP	single nucleotide polymorphism
SSCP	Single Strand Conformation Polymorphism
STR	short tandem repeat
STS	sequence tagged site
T	thymidine
U	uridine

Chapter One: Background

Hearing loss is a common sensory disorder in human populations. Approximately 70 million people worldwide suffer from a hearing loss exceeding 55dB (Kubisch, et al., 1999), with many more having moderate to severe hearing loss greater than 30 dB (Stein, 1999). In the United States, approximately 1 child in a 1000 is born profoundly deaf (Morton, 1991). Overall approximately 28 million Americans experience hearing loss, and more than 2 million of them are profoundly deaf.

Excluding presbycusis about 50% of hearing loss is thought to have a genetic basis; however, that percentage is increasing as we make progress in reducing the incidence of acquired deafness (Giersch, et al. 1999). Of the hearing-loss disorders attributable to genetic causes, ~70% is classified as nonsyndromic and the remaining 30% as syndromic. Of the nonsyndromic hearing loss, ~77% is autosomal recessive, 22% is autosomal dominant, 1% is X-linked, and <1% is due to mitochondrial inheritance (Morton, 1991).

Hearing loss is a handicap that has an important impact on the educational and social development of affected individuals, especially if it is diagnosed too late (Desai, et al. 1995). For the elderly, it increases vulnerability and limits the quality of life (Giersch, et al. 1999). Presbycusis is an age-related hearing loss, which is thought to be due to a combination of genetic and environmental causes (Fischel-Ghodsian, 1999).

A. Mechanism of normal hearing

The human auditory system is composed of three anatomical compartments: the outer, middle, and inner ears. The outer ear is the part that can be seen and includes the ear canal. The middle ear includes the tympanic membrane and the three small bones or ossicles. The eustachian tube connects the middle ear to the throat and helps equalize pressure in the middle ear with air pressure.

The inner ear includes the cochlea and the semi-circular canals and provides two sensory systems: the auditory system for hearing and the vestibular system for spatial orientation and equilibrium. It consists of the bony and the membranous labyrinths. The bony labyrinth is filled with a fluid called perilymph and contains three major cavities: the vestibule, the cochlea, and the semicircular canals with the sacculus and the utriculus. The membranous labyrinth containing endolymph consists of an elaborate series of communicating ducts and sacs immersed in the perilymph of the bony labyrinth. The three semicircular ducts, the sacculus, and the utriculus form the membranous part of the vestibular apparatus, while the cochlear duct, which contains the hair cells of the organ of Corti, forms the membranous part of the cochlea.

The semi-circular canals are the balance organs, they respond to rotatory acceleration, and the utriculus and sacculus respond to linear acceleration. The vestibular apparatus converts acceleration into electrical impulses that are combined with information from the visual and proprioceptive system to define the sense of equilibrium. The cochlea receives and processes auditory signals, It looks like a snail shell with two and half coils.

Inside the cochlea are sensory cells (hair cells) in the organ of Corti that respond to sound and send nerve signals to the brain.

The organ of Corti, residing between the tectorial and the basilar membranes, contains two types of sensory cells: a row of inner hair cells (IHCs) and three rows of outer hair cells (OHCs). The IHCs are pure receptor cells that transmit signals to the acoustic nerve and the auditory cortex. The OHCs have both sensory and motor elements that contribute to hearing sensitivity and frequency selectivity by amplifying sound reception. The relative movement of the tectorial and the basilar membranes leads to deflection of the stereocilia of the IHCs and OHCs. This movement results in an influx of potassium ions into the hair cells through channels at the tip links of the stereocilia, leading to stimulation of the underlying nerve cells. The nerve cells then convey the auditory signal to the auditory cortex. Thus the hair cell acts as a mechanoelectrical transducer producing an electrical signal that is transmitted through nerve fibers and the spiral ganglion to the cochlear nerve and the auditory cortex of the brain.

The elongation and contraction of the OHCs caused by the acoustical stimulation involves concerted action of many motors along the plasma membrane and the cytoskeletal lattice, which consists of filamentous proteins such as actin. Movements of tip links are controlled by myosins.

The movement of the tip links opens the transduction channels, which leads to an influx of endolymphatic K^+ into the sensory hair cells resulting in cell depolarization. Depolarization of the hair cells activates calcium channels on the basolateral side of the

cells, resulting in calcium influx into the hair cells. This influx triggers the release of the neurotransmitters that activate the acoustic nerve. The hair cells are repolarized when potassium ions leave these cells through potassium channels and enter the epithelial supporting cells. The potassium ions then diffuse to the stria vascularis through gap junctions formed by connexins and are secreted back into the endolymph through potassium channels, thereby resetting the mechanoelectrical transduction system (Willems, 2000, Markin, et al. 1995, Hudspeth, et al. 1989, Nobili, et al. 1998).

In summary, a sound captured by the external ear sets in motion ossicles in the middle ear; these, in turn, create pressure fluctuations in the cochlear fluids that cause a displacement wave to propagate along the basilar membrane. The displacement wave stimulates cellular transducers, the inner and outer hair cells (IHCs and OHCs), to generate electrical receptor potentials that mimic the acoustic stimulus. Finally, these receptor potentials produce chemically mediated excitation in the peripheral terminals of cochlear afferent neurons, generating trains of electrical pulses (action potentials) that travel via the auditory nerve to the cochlear nucleus, the first station of the auditory central nervous system, and finally to the auditory cortex of brain (Petit, 1996).

B. Measurement of ear hearing function

For both research and treatment it is important to differentiate normal hearing from impaired hearing. A standard hearing test battery typically includes (1) puretone air and bone conduction testing, (2) speech audiometry, and (3) acoustic immittance measures

including typanometry and acoustic reflexes. Results of the pure-tone air and bone conduction testing are plotted on a chart called an audiogram. Results from air conduction testing identify the degree and configuration of hearing loss. Comparison of thresholds for air and bone conduction can determine whether hearing loss is conductive, sensorineural or mixed. Speech audiometry measures an individual's ability to recognize words and/or sentences. Two speech audiometry measures are included in the basic evaluation: the speech recognition threshold (SRT) and suprathreshold word recognition measure. The SRT provides a check on pure-tone thresholds and, in general, should agree with the pure-tone average (PTA) of 500, 1000, and 2000 Hz from air conduction. The type and degree of hearing loss, and pathologic conditions or disease processes can influence speech recognition scores. Individuals with pure conductive hearing loss score within the excellent range, since only the intensity of sound is affected. Individuals with sensorineural loss may score poorly on word recognition measures even at high presentation levels since hearing loss prevents them from receiving all the sounds within the word. The acoustic immittance measurement evaluates the middle ear status (Browning, 1998).

Measures of Otoacoustic emissions (OAE) and Auditory Brainstem Response (ABR) are the two other tests used to discriminate between cochlear and retrocochlear types of hearing loss. It is thought that physical movement of the outer hair cells generates otoacoustic emissions during the normal cochlear transduction process. This indicates proper cochlear function. The OAE test tests the function of OHCs by detecting and measuring these emissions. An ABR test measures electroencephalographic waves

normally generated by the cochlea in response to a sound stimulus and sent to the brain through the cochlear nerve.

There is no internationally accepted definition of what constitutes a significant hearing loss (Davidson, et al. 1988). A variety of different criteria have been used to report hearing loss severity. According to the definition of hearing loss from American National Standards Institute (Grundfast, et al. 1999), audiometric zero is a set of values of hearing level that correspond to the average detection of sound at a range of signal frequencies, for example, 500 Hz, 1000Hz, 2000Hz, and so forth. Individuals generally are considered to have normal hearing if their ability to detect sound falls within 0 and 15 to 25 dB HL. The general hearing loss categories used routinely by most hearing professionals in their practice are described as follows: "mild" hearing loss is described as detection of sound within the 15 to 30 dB HL range, "moderate" hearing loss within 31 to 60 dB HL, and "severe" hearing loss 61 to 90 dB HL, "profound" hearing loss is 90 dB HL or greater (Carney, et al. 1998).

Hearing loss can be described according to several different criteria: it can be prelingual or postlingual based on the age of onset; mild, moderate, severe, or profound, based on the severity; unilateral or bilateral based on the affected ear(s); nonsyndromic or syndromic based on presence of other symptom(s); genetic or acquired based on etiology; sensorineural, conductive, or mixed based on the type of pathology; temporary or permanent; stable, progressive or fluctuating depending on the stability. Overall the most common type of hearing loss is the bilateral, nonsyndromic, permanent, progressive,

stable, sensorineural hearing loss associated with increasing age. This is usually assumed to an acquired, although genetic factors appear to contribute (Rehm, et al. 1999).

C. Hearing loss research

Before the concepts of the modern genetics, heredity was generally accepted as a cause of hearing loss since the 19th century (Cremers, 1995). Research into the causes of hereditary hearing loss was first initiated in the second half of the 19th century. At this time several common hereditary syndromes that include hearing loss as a characteristic symptom were reported (Reardon, 1992). Our knowledge of syndromic and nonsyndromic forms of hereditary hearing loss has greatly increased over the last half of the 20th century. Nowadays, since possible preventive and /or therapeutic approaches depend very much on the knowledge of the gene product, it is no longer sufficient simply to show that a disease is inherited in an autosomal or sex-linked recessive or dominant pattern. It is necessary to know the product of the mutated gene and the mechanism by which it produces the syndrome.

Prior to 1994, only three loci for nonsyndromic hearing impairment (NSHI) had been identified. In the late 1980s, a sex-linked form of NSHL, DFN3, was mapped to Xq21.1 by linkage analysis in Mauritian kindreds (Brunner, et al. 1988; Wallis, et al. 1988). In 1992, an autosomal dominant locus, DFNA1, was mapped to 5q31 by linkage analysis in

Table 1. Summary of the cloned hearing loss genes

Gene function	Gene	Locus	Reference
Cell-cell communication			
Gap junction protein, pottassium recycling	GJB2 (Cx26)	DFNA3 DFNB1	Kesell, et al., 1997
Gap junction protein	GJB6 (Cx30)	DFNA3	Grifa, et al., 1999
Gap junction, potassium recycling	GJB3 (Cx31)	DFNA2	Xia, et al., 1998
Potassium channel, potassium recycling	KCNQ4	DFNA2	Kubisch, et al., 1999
Anion transporter, Pendrin	SLC26A4	DFNB4	Li, et al., 1998
Structural genes			
Unconventional myosin (molecular motor)	MYO7A	DFNA11 DFNB2	Liu, et al., 1997a Liu, et al. 1997b; Weil, et al., 1997
Unconventional myosin	MYO15	DFNB3	Wang, et al., 1998
Muscular motor, unconventional	MYO6	DFNA22	Melchionda, et al.,2001
Cellular myosin, heavy chain 9, nonmuscle	MYH9	DFNA17	Lalwani, et al., 2000
a-tectorin (tectorin membrane matrix protein	TECTA	DFNA8/12 DFNB21	Verhoeven, et al., 1998 Mustapha, et al., 1999
Extracellular matrix, maintenance of cochlear cell	COCH	DFNA9	Robertson, et al., 1998
Collagen, structural protein in cochlea	COL11A2	DFNA13	McGuirt, et al., 1999
Tight junction claudin-14	CLDN14	DFNB29	Wilcox, et al., 2001
Stereocilin, stereocilia structure	STRC	DFNB16	Verpy, et al., 2001
Hair bundle formation,	CDH23	DFNB12	Bork, et al., 2001
Transcription factors/developmental regulators			
Transcription factor	POU3F4	DFN3	De Kok, et al., 1995
Transcription factor	POU4F3	DFNA15	Vahava, et al., 1998
Transcription activator	EYA4	DFNA10	Wayne, et al., 2001
Miscellaneous			
Transmembrane protease, serine-3	TMPRS3	DFNB8/10	Scott, et al., 2001
Diaphanous(cytokinesis and cell polarity)	HDIA1	DFNA1	Lynch, et al., 1997
Cochlear hair cell function	TMC1	DNNA36 DFNB7/11	Kurima, et al, 2002
Trafficking of membrane matrix protein	OTOF	DFNB9	Yasunaga, et al., 1999
Otoancorin, an inner ear protein	OTOA	DFNB22	Zwaenepoel, et al., 2002
Wolframin, unknown function	WFS1	DFNA6/14	Bespalova, et al., 2001 Young, et al., 2001
Unknown function	ICERE-1	DFNA5	Van laer, et al., 1998
Dentin phosphoprotein	DSPP	DFNA39	Xiao, et al., 2001
Harmonin	USH1C	DFNB18	Ahmed, et al 2002
Mitochondrial genes			
Mitochondrial ribosome RNA	12s rRNA	1555A>G	Prezant, et al., 1993
Mitochondrial tRNA	TRNA Ser (UCN)	7445A>G	Reid, et al., 1994

Adapted from the hereditary hearing loss homepage (www.uia.ac.be/dnalab/hhh). DFNA indicates dominant inheritance. DFNB indicates recessive inheritance, DFN indicates X linked inheritance.

a large, extended Costa Rican kindred (Leon, et al.1992). In 1993, a third locus, a mitochondrial A → G mutation in a highly conserved region of the 12S rRNA gene (nt1555) was identified in a large Arab-Israeli pedigree with maternally inherited NSHI (Prezant, et al. 1993).

In the last decade the advances in molecular biology, such as positional cloning, have provided tools to identify the genes that function in the hearing process. To date, more than 80 nonsyndromic sensorineural hearing loss loci have been mapped, 41 loci for dominant, 36 for recessive, 8 for X-linked, 1 for modifier, and 6 for mitochondrial hearing loss. Twenty-eight of these genes have been cloned and their sequences determined (http://www.uia.ac.be/dnalab/hhh/) (Table 1).

In view of the complex structure of the inner ear and the mechanisms of normal hearing, it is not surprising that changes in hundreds of different genes can result in hearing impairment. The genes associated with hearing loss encompass a variety of functions ranging from extracellular matrix, cytoskeletal, ion channel, synaptic vesicle trafficking proteins to transcription factors. Mutations in any of these genes can cause hearing loss.

It is also interesting to note that (table 1) some of the identified hearing loss genes are responsible for both autosomal dominant and autosomal recessive forms of deafness (e.g. MYO7A, GJB2, TECTA, TMC1) (Petersen, 2002). Some are responsible for both syndromic and non-syndromic deafness (e.g. MYO7A, WFS1, EYA4, MYH9, DSPP) (Petersen, 2002, Xiao, et al., 2001). Also in several cases different mutations in a single

gene can lead to congenital or late onset, recessive or dominant, mild or profound hearing loss (GJB2, MYO7A, TECTA) (Lynch, et al., 1997, Liu, et al., 1997a, Liu, et al., 1997b, Verhoeven, et al., 1998).

D. Background of the MSUDF1 family

In the spring of 1996, the MSUDF1 family with adult-onset progressive, sensorineural hearing loss was referred to the MSU hearing research group. Research was begun to identify the genetic cause for hearing loss in this family.

This MSUDF1 family is an extensive kindred of English descent, whose ancestors immigrated to the mid-Michigan area at the beginning of 20^{th} century from Cornwall, England. In the three generations now living, several members have hearing impairment (Figure 1, Figure 7). The proband (306) was 65-year old women with profound hearing loss identified by a local Audiologist in 1996. She has six children and five of them are affected with the same form of hearing loss. She reported that both her father and grand mother developed severe hearing loss. The English census for 1871 recorded her great great grand father as deaf. He was born in 1806 and was aged 65 years at the time of the census. No dysmorphology was observed in the proband or her affected sibling and children.

There are several strategies to identify human disease genes: (1) functional cloning which starts with a known protein for which there is biological evidence of a role in a certain genetic disease. (2) candidate gene cloning, which starts with a known gene of function

possibly related to a certain genetic disease. (3) positional gene cloning, which starts with a subchromosomal location, and construction of physical and genetic maps of the region. (4) positional–candidate gene cloning, which entails mapping a disease gene of unknown function to a subchromosome location, and then examining up-to-date genetic and transcript maps to determine which coding region (genes, ESTs) have been mapped to the same interval as the disease. With more and more human genes being mapped to specific subchromosomal regions, positional-candidate gene cloning is now the most common approach in the field of identifying disease genes.

An important tool for finding a hearing gene is by linkage study in a large family, in which the hearing process of some family members is disrupted. Linkage is suspected when a gene marker and the phenotype cosegregate. In such families, the causative gene can be mapped and identified, so that useful information about its expression pattern, function and role in hearing process can be ascertained.

The MSUDF1 family (figure 1 & figure 7) is such a family in which nonsyndromic sensorineural hearing loss develops in young adults. Phenocopies, especially in late onset hearing loss where environmental factors might generate a similar phenotype, and reduced penetrance, where an individual with same genotype might fail to express it, can interfere with the linkage analysis. The individual 307 is defined as phenocopy because he had experienced considerable noise exposure and had a somewhat different hearing loss pattern (refer to chapter one, figure 4 and figure 7). By age 20, there is apparently complete penetrance of the hearing loss in this family since there is a nearly 1:1 ratio of affected to unaffected offspring of affected parents.

1. Definition of the phenotype in MSUDF1 family

For the family's convenience, audiologic testing for this family was initially performed on about 45 family members at the proband's home during a family reunion. The test protocol for all subjects included otoscopy, tympanometry and puretone air conduction screening at octave and interoctave intervals from 1000 – 8000 Hz under headphones. Unmasked puretone air and bone conduction thresholds were obtained for all individuals who failed to detect one or more of the puretone stimuli. Background noise levels precluded threshold search below 20 dB HL and frequencies below 1000 Hz. Subjects who showed greater than 20 dB hearing loss were later retested in MSU's Oyer Speech – Language-Hearing Clinic in audiometric testing booths. Some family members were retested several times at intervals of one to two years.

The clinical phenotype of hearing loss in this family is somewhat similar to age-related hearing loss, or presbycusis but with an earlier onset. Thus identification of this hearing loss gene may help to understand the etiology of presbycusis. Knowledge about the gene will help to enhance not only the understanding of the molecular basis of auditory transduction, but also the understanding of the pathological process involved in hearing loss. This knowledge could perhaps lead to treatment or prevention of some forms of presbycusis.

Results of the ascertainment of the phenotype in this family in detail are in chapter two.

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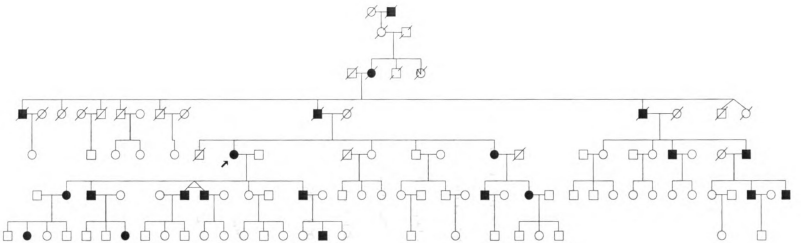

Figure 1. Pedigree of MSUDF1 family with dominant pattern of inheritance. Individuals are defined as affected if their audiograms show drop-off in high frequencies (details for age see Figure 11).

2. Gene mapping using whole genome scan and haplotype analysis

To determine whether the gene responsible for hearing loss in MSUDF1 family is linked to an already identified locus, sixteen informative family members were tested to look for linkage to DFNA1-15 and DFNB3-8 (http://hgins.uia.ac) (Van Camp and Smith, 1997a). No linkage was found. We therefore concluded that MSUDF1 represents a new hearing loss gene with an unknown locus. To look for linkage to a known marker, a genome-wide screen was conducted in the Laboratory of Dr. Thomas Friedman in the summer of 1998. The Weber 8A marker set (Research Genetics) was used. This ABI Prism™ Linkage mapping Set Weber 8A consists of 45 panels of primers to amplify selected loci, Each panel has 8- 10 fluorescently-labeled primer pairs; in total, 387 markers are represented, 89% of which are either tri- or tetra-nucleotide repeats with an average heterozygosity of 0.76. The average spacing between microsatellite markers is 10 cM meaning that the average maximum distance between the new gene and marker would be 5 cM.

Microsatellites, also known as short tandem repeats (STRs) or simple sequence repeat (SSR), referring to a stretch of tandemly repeated nucleotides mapped to a specific region, are polymorphic, easily analyzed and occur regularly throughout the genome, making them especially suitable for genetic analysis. Variation in the number of tandemly repeated units results in highly polymorphic banding patterns. The repeated unit can be a mono-, di-, or tetra-nucleotide with di-repeats being the most common.

Multiplex PCR was optimized for each of the three fluorescent dyes (Tet for green; Fam for blue; and Hex for yellow) in each of the panels and typed in this family to look for positive linkage to a unique chromosome region.

After positive linkage was found, haplotype analysis using additional microsatellite markers in the region was done to look for the meiotic breakpoints. The breakpoints for D17S914 and D17S668 were found on individuals, 511 and 421. As a result we were able to map the hearing loss gene in this MSUDF1 family to a 5.5 cM locus on 17q25. It was designated DFNA20 by the nomenclature committee of the Human Genome Organization. DFN indicates that the gene is for hearing loss (deafness), A indicates that the inheritance pattern is autosomal dominant and 20 indicates it is the twentieth gene among mapped genes of the same type.

To more closely define the interval containing the gene, examination of additional chromosomes is required to increase the possibility of recombination. Therefore, more family members will be needed to establish closer breakpoint. A branch of this family in England was found with four individuals who claimed that they have hearing loss. We expected they would share the same haplotype as the MSUDF1 family and might provide a closer breakpoint. However, the genotyping result of the four individuals from England showed that they do not share any haplotype with MSUDF1 family. Audiometric testing also indicated that their hearing loss was very different from that of the Michigan family, We therefore concluded that the MSUDF1 hearing loss gene was not present in this branch of the family.

In order to convince ourselves that we have identified the DFNA20 gene, observation of other mutations in the same gene that also cause hearing loss would provide additional confirmation. Other families with similar hearing loss phenotypes or with an overlapping locus for a hearing loss gene might provide such confidence. We anticipated that they might share the same haplotype with MSUDF1 family if the same mutation is causing hearing loss. However they might have a different haplotype if there are different mutations in the same gene or mutations in different genes in the same interval.

We therefore looked for families whose hearing loss mapped to the same chromosomes locus. Family 1681 from Boys Town Hospital is a family with dominant progressive hearing loss. It was brought to our attention by a family member who read our paper describing DFNA20. Configuration of their hearing loss and the mode of inheritance is similar to that of MSUDF1 family.

Two additional families (DFNA26) were identified, they mapped to an overlapping chromosomal region and were collected by Dr. Richard Smith (Yang & Smith, 2000). We did not know if the DFNA26 families represented the same gene as the MSUDF1, or if they represent an additional gene at a nearby locus. We shared all the markers to analyze the haplotype information.

Detailed results of this study are reported in chapter three.

3. Analysis of a mouse model for DFNA20 family

The mouse and human auditory systems have many similarities. This suggests similar genetic involvements in the development of the ear in the two species (Steel and Brown, 1994). The mouse is frequently used as a model for hearing loss in mammals and has provided a powerful tool for the identification of human hearing loss genes (Probst and Camper, 1999). There are many mouse models for hearing impairment (Steel and Brown, 1994) (http://hgins.uia.ac.be/dnalab/hhh). One of these, the Jackson shaker (js) demonstrates circling, headshaking, hyperactivity in addition to deafness. The js locus was mapped to mouse chromosome 11 in a region that has homology of synteny to human 17q25 (http://informatics.jax.org). A putative gene for js has been identified and described as a motor protein (kinesin). A mutation was found in this gene in the js mouse (Kikkawa, et al.1998). Homozygous js/js mice show incomplete differentiation of the stereocilia of the outer hair cells and lack the W-configuration of the stereocilia because there is disarray and deletion of the stereocilia (Kitamura, et at., 1992). No abnormalities were found in heterozygous mice but these were studied only until day 30 postpartum (Kitamura, et al., 1992). Another mouse mutant (designated "new-mutant"), which maps to the same region and is thought to be allelic, displayed a similar pathology. Morphological examination of the new-mutant homozygotes from ages 10 days to 18 months showed an age-dependent degeneration of the outer hair cells from the basal to apical part of the cochlea (Kitamura, et al., 1991a and b). We established collaboration with Kikawa group (Kikkawa, et al.1998). Primers were designed to screen a BAC library and typed in G3 and TNG RH panels. This study is detailed in chapter four.

4. Analysis of the candidate genes with known function(s) in DFNA20 family

At present, the most efficient way of looking for a disease-causing gene is to look at the known genes in a region to which it maps. To begin with, a candidate gene was defined as a gene that had both a possible role in the hearing process and that mapped to the defined region. DFNA20 was localized to 17q25 to a region of 5.5 cM with an approximate physical distance of 2.5 megabases. This incompletely sequenced region contains a number of possible candidate genes (table 4). Analyses of these genes are detailed in chapter five.

5. Refinement of the DFNA20 critical interval and construction of the physical contig across the region

The DFNA20 interval is in the telomeric region of the long arm of chromosome 17, in which the reported sequence is very incomplete. Once the mouse model and the mapped genes had been excluded, it became clear that further refinement of the DFNA20 interval and construction of a physical contig across it were necessary. Additional SNP and microsatellite markers were generated in order to look for closer breakpoints.

a. Generating markers:

To develop the markers required for narrowing the interval in this family, two major types of polymorphic markers were exploited, microsatellite and single nucleotide polymorphisms (SNP).

SNPs have only 2 alleles, therefore the maximum heterozygosity possible is 50%. Because SNPs are less heterozygous they are likely to be less informative than SSRs and are used as markers only when no SSRs are readily available. SNP studies focused on individuals 409 and 421 (non-affected and affected individuals with recombination flanking the critical region), 511 (non-affected by age of 23), and 306 (affected proband). In the absence of genomic sequence, the 3'UTR sequence of cDNA is predicted to be the least conserved and therefore the most likely to harbor SNPs. The 3' sequences of ESTs already mapped in the critical region were aligned and examined for potential SNPs. Potential SNPs were chosen based on the following criteria that (1) The variant was found in more than one EST, (2) It was not in a region of potential sequence error such as runs or gaps, (3) It was not in areas where there were N's in the sequence harboring the variant.

b. Screening of BAC Library

The Research Genetics provides a human BAC library (www.tre.caltech.edu) as PCR ready plasmid DNA arranged in an easy to screen format. This arrayed human genomic BAC library with approximately 4 x converage is represented by 96,000 BAC clones with average insert size of nearly 140 kb. "It serves to integrate genetic, STS, and

cytogenetic map information while providing direct access to stable material that may be utilized for the purpose of molecular analyses, medical diagnostics, and for genomic sequencing" (Kim, et al, 1996).

Using PCR strategy based on the STSs and sequenced BACs end sequence to screen BAC library, it should be possible to pull out the BACs that are aligned with each other and contain specific STSs. We expected that this approach would help to bridge the gaps on the physical contig across DFNA20 interval.

c. Searching databases of the Human Genome Project and Celera

The HGP (http://www.ncbi.nlm.nih.gov) uses the hierarchical shotgun sequencing approach to sequence the human genome. This approach involves generating and organizing a set of large-insert clones (typically 100-200 kb each) covering the genome and separately performing shotgun sequencing on appropriately chosen clones (International Human Genome Sequencing Consortium., 2001). The Celera genomics company (http://www.celera.com) uses a mixed strategy, involving combining some coverage with whole-genome shotgun data generated by the company together with the publicly available hierarchical shotgun data generated by the International Human Genome Sequencing Consortium (Venter, et al. 2001). The sequencing data of human genome are pouring into the public area even though the draft sequence from Celera is less freely available. We took advantage of these two databases while assembling our physical contig.

d. Constructing the physical map across the DFNA20 critical region

To build up a BAC contig across the critical region, 60 STSs in this interval (http://www.ncbi.nlm.nih.gov) were used to search the HGP and Celera database in order to find any sequenced BACs and genomic scaffold. Microsatellite markers were looked for on the sequenced BACs and typed in the MSUDF1 family to find out if those BACs were inside or outside the interval. Those sequenced BACs and their end sequences were used to search Genome Survey Sequence (GSS), High Throughput (HTGS) and Celera database (http://www.publication.celera.com) and obtain more aligned BACs. Meanwhile the primer pairs for each of the STS mapped to the critical region between D17S914 and D17S668 (http://www.ncbi.nlm.nih.gov) were ordered to screen the personalized BAC library (Research Genetics) (table 8). The ends of any STS positive BACs were then sequenced and used to search for other BACs in order to extend the physical contig. Also the STS primer pairs are used to type those BACs from the databases only with end sequence to see which STSs hit the BACs.

Both SNPs and microsatellite markers were used to decide which BAC is linked to DFNA20 phenotype, and which is outside of the critical interval.

Results are detailed in chapter six.

6. Analysis of interesting transcripts in the DFNA20 critical interval

Because we are studying a dominantly inherited disease only one of the two alleles is expected to carry a mutation so that in cells derived directly from an affected family member there will always be a normal sequence present. To make sure that we will not miss a heterozygous mutation, especially a small deletion, we decided to generate somatic cell hybrids with the normal and affected chromosomes in separate lines. GMP Genetics, Inc. (www.gmpgenetics.com) performed this service for us. GMP conversion technology™ employs fusion between human and rodent cells to create hybrids that contain only one copy of a human chromosome. We have three cell lines provided by GMP, each one is supposed to contain one copy of chromosome 17 of the proband 306.

The Human Genome Project has not identified or annotated any human or non-human mRNAs deriving from the DFNA20 critical region although there are EST and novel mRNA transcripts in the region. To identify interesting transcripts, firstly the STS sequences found in the BACs in the interval were used to probe a human fetal cochlear cDNA phage library made at NIDCD and provided by Dr. Robert Morell. Meanwhile a PCR-based method was used to screen this phage-cDNA library in order to get the corresponding cochlear clone of certain STS (Israel, 1993).

For an STS not positively identified in human cochlear library, we looked for its mouse unigene cluster to find out if the mouse homolog is expressed in mouse inner ear using the Inner Ear Gene Expression Database from the Corey lab in Harvard Medical School

(http://www.mgh.harvard.edu/depts/coreylab/genomics.html). This database gives the expression profile for the mouse cochlea and has over 30,000 genes and ESTs.

Interesting transcripts –newly identified by the HGP- were selected for study chosen because they lay within the physical contig and because of their potential cochlear function. Using the UCSC genome map (http://genome.cse.ucsc.edu), ESTs mapped to this region were first identified. The corresponding I.M.A.G.E clones (cDNA clones) were then obtained and the longest inserts that contained the EST of interest were sequenced in order to obtain the full-length cDNA. Based on northern blot hybridization, the profile of the size and tissue expression of these genes were determined.

In order to compare the gene on affected and unaffected chromosomes, primers were designed from the cDNA sequences or/and genome sequences of these genes. The cDNA or/and DNA of the two different hybrids were amplified. The sequences of the two hybrids for each of these genes were compared in order to search for a significant heterozygous change.

Analyses of these four interesting transcripts are described in chapter seven.

Chapter Two: Description of the MSUDF1 Family and Hearing Test Results

A. Introduction

MSUDF1 (figure 1 and figure 7) is a three-generation family from the Mid-Michigan area. Audiologic testing for this family was initially performed on about 45 family members at home during a family gathering organized by the proband. Follow-up testing is continually done for the younger generation in MSU's Oyer Speech –Language-Hearing Clinic in audiometric testing booths (see chapter one).

The locus on 17q25 responsible for MSUDF1 family was named officially DFNA20 by the nomenclature committee of the Human Genome Organization in 1999.

B. Results

The proband first noticed hearing loss at about 30 years old. She was severely affected with hearing loss by age 65 when she came to us. Although she was fitted with bilateral hearing aid, she had difficulty communicating. Pure-tone air and bone conduction thresholds from 250 – 8000 Hz in 1997 revealed bilateral mild to profound, sloping sensorineural hearing loss , with no response at the limits of the audiometer at or above 6000 Hz in either ear (Figure 2). Physical exam by a clinical geneticist revealed no dysmorphology. A neurological exam showed no evidence of problems with balance or vision. The hearing loss was therefore considered to be nonsyndromic.

The proband has six children, five of whom have the same type of adult onset hearing loss. The audiograms of the proband (306) and her son (406/407) define a mature-onset progressive, bilateral, sloping, sensorineural hearing loss (Figure 2 & 3).

The proband's husband (307) who also had severe hearing loss experienced his hearing loss beginning around 50 years old. He has had a great deal of noise exposure including driving a tractor without ear protection and recreational shooting. The pattern of his hearing loss is somewhat different from other family individuals (Figure 4). In this study he is defined as a "hearing" person because his loss is thought to have a different etiology than other family members (Elfenbein, et al. 2001).

The grandchildren of the proband have been tested repeatedly and some of them show a clear loss in higher frequencies of 6000, 8000 Hz. The progression of hearing loss appears to resemble presbycusis most closely at the early stages, with a steep drop off in the higher frequencies (Figure 5). Because her genotype defined a possible breakpoint, follow-up testing of hearing for individual 511 was needed in order to define her phenotype clearly.

The retesting of individual 511 was done in Oyer Speech –Language-Hearing Clinic at MSU, using a newly acquired high frequency audiometer. She showed no sign of loss at any frequency by age of 23 (Figure 6). Therefore she was classified as a hearing individual from this family for the linkage study.

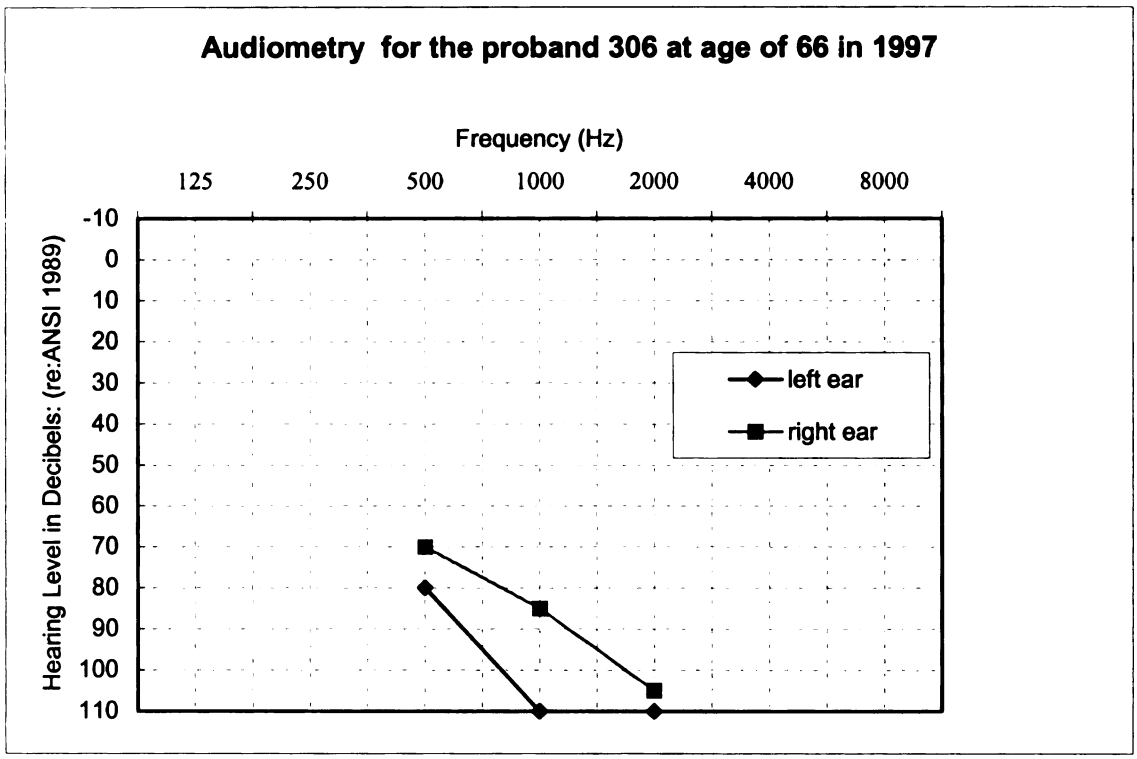

Figure 2. Audiometry for the proband (306) at age of 66. It shows a bilateral, sloping, profound hearing loss.

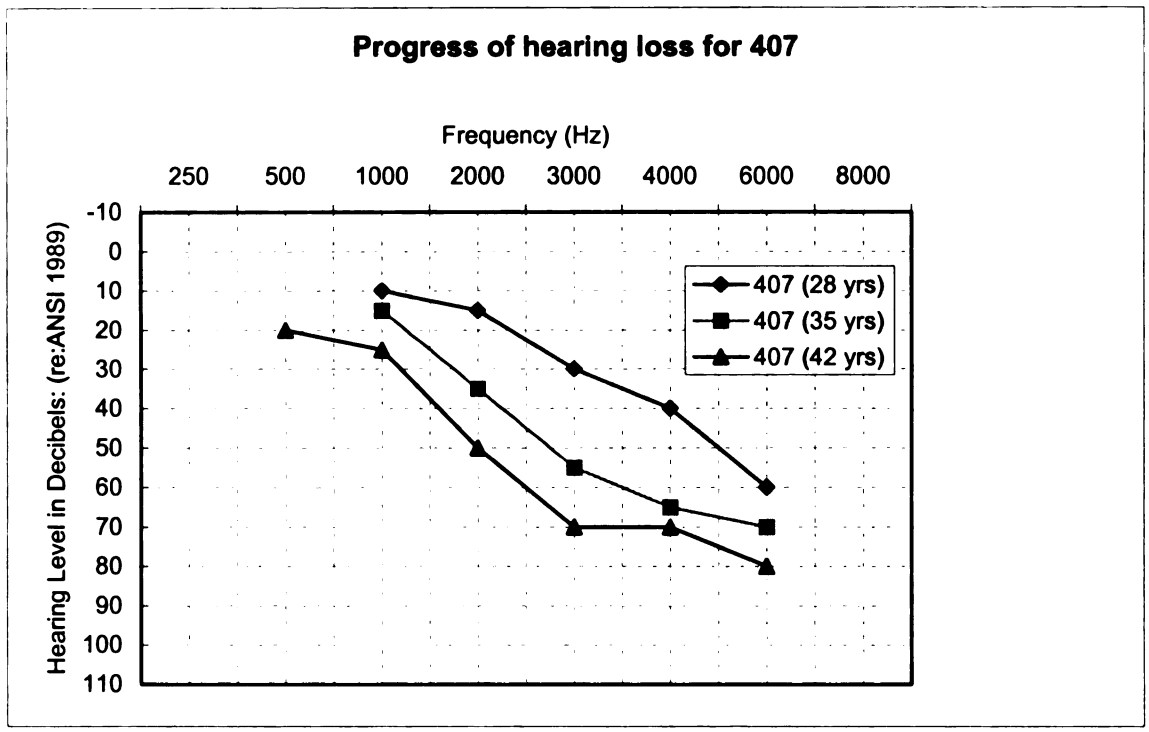

Figure 3. Progress of hearing loss for family member 407. It shows a progressive hearing loss over time. The audiometries from which this figure was summarized were provided by the individual's employer.

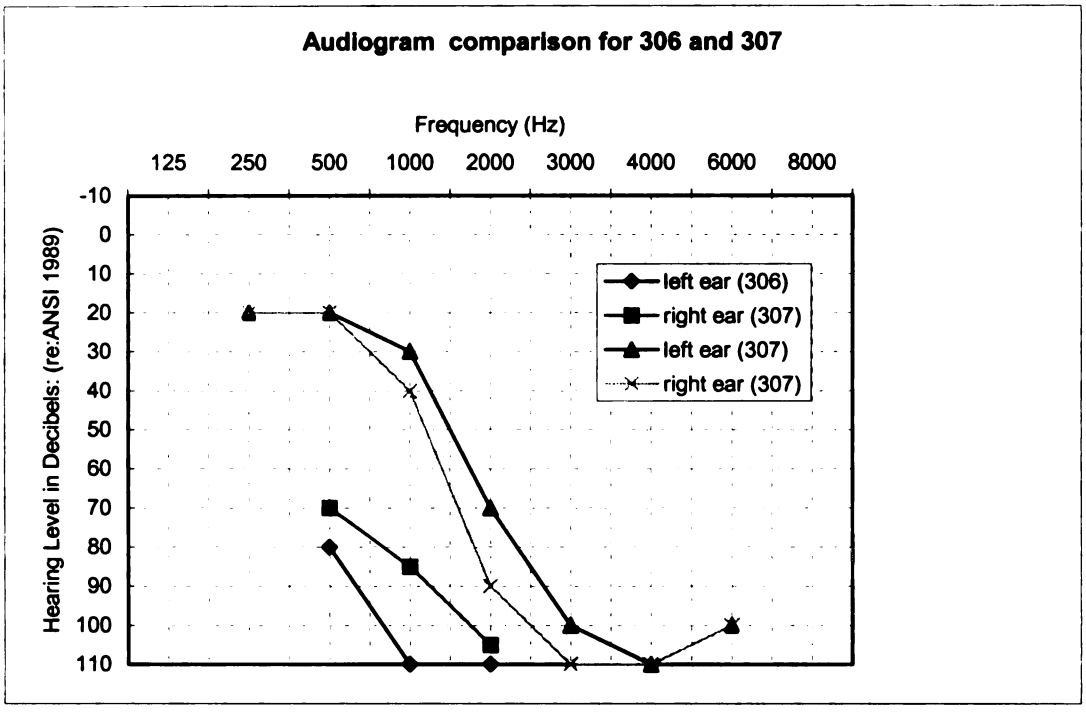

Figure 4. Audiometric comparison for the proband 306 and her husband 307. The patterns of their hearing loss are different. The audiogram for 307 shows that his hearing at lower frequencies up to 1000 Hz is still somewhat conserved instead of a corner audiogram like 306.

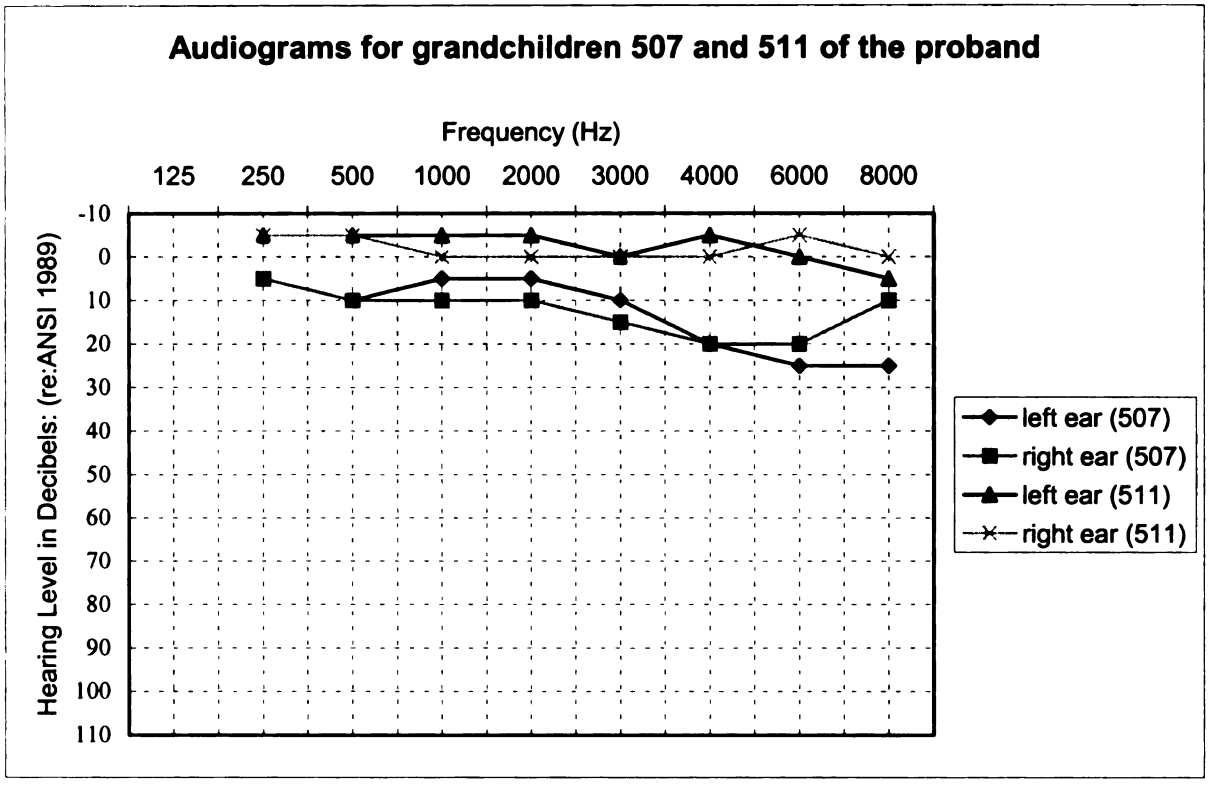

Figure 5 Audiometric comparison for affected individual 507 at age of 13 and unaffected individual 511 at age of 21. The audiogram of 507 shows she is going to be affected, as her hearing starts to drop off at high frequency of 3000 Hz. The audiogram for 511 shows she is still in the normal hearing range at age of 21.

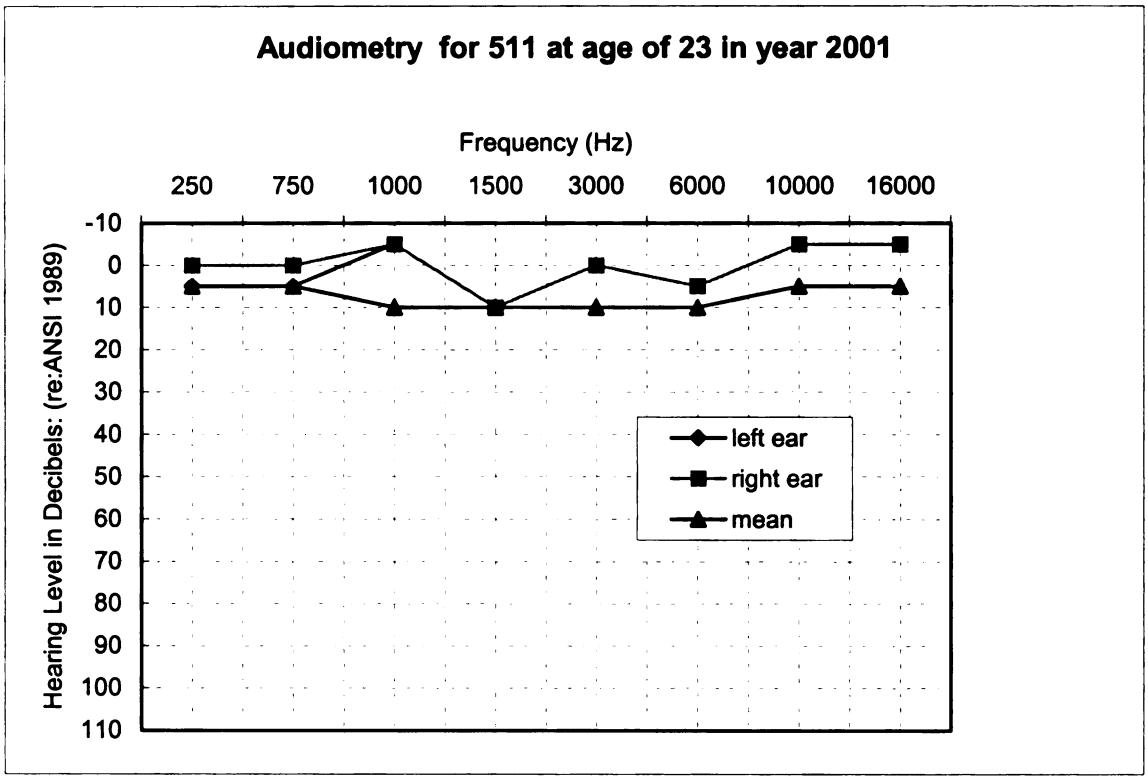

Figure 6. Follow-up audiometry for 511 at age of 23 in year 2001. The mean is the average hearing for young adult high frequency thresholds (18-26 yrs). This figure shows hearing for 511 is still in the normal range of hearing at age 23.

1. Discussion

The inherited hearing loss demonstrated by this mid-Michigan family of English descent is a form of nonsyndromic, mature-onset, bilateral and progressive, hearing loss. Family history indicates an autosomal dominant pattern of inheritance with virtually 100% penetrance by middle age.

Affected members have a bilateral, sloping, progressive, sensorineural hearing loss showing first at 6000 and 8000 Hz although they were unaware of it. Hearing loss could be identified in some family members in their early teens but was clearly evident by the early twenties. The degree of hearing loss increases with age, so that threshold shifts are eventually seen at all frequencies. A sloping configuration is maintained. Subjects generally became aware of their hearing loss in their early thirties.

Because hearing loss in the high frequencies is also characteristic of noise-induced trauma, threshold patterns demonstrated by some of the younger individuals (<25 years) were difficult to phenotype. Ambiguous individuals were not used in the initial screens for linkage.

The age of onset of measurable hearing loss at around 20 years in the family is later than most of the autosomal dominant forms of hearing loss so far described (http://dnalab-www.uia.ac.be/dnalab/hhh/). The pattern of hearing loss and the progress resemble the most common sensory form of presbycusis but shifted earlier by about 30 years. The rate of change at 4,000 Hz is on the order of 2 dB loss /year.

With our present data, it is impossible to tell for certain whether the DFNA20 hearing loss has an intrinsic cause, such that the mutated gene itself causes disruption and/or death of the cells of the sensorineural apparatus, or whether the mutation merely causes particular sensitivity to environmental assault. However, the appearance of a steady progression with age when all affected family members are compared and the high degree of penetrance evidenced by the 1:1 ratio of affected to unaffected family members suggests that the former is most likely.

Presbycusis is a common and disabling phenomenon of aging. In the general population it is thought to be due to combinations of both genetic and environmental causes. Identifying the genetic elements, whether intrinsic or predisposing, would be difficult without likely candidates. Since the hearing loss in this family resembles that which is associated with presbycusis, identification of the DFNA20 gene may well provide information about the genetic etiology of presbycusis.

Chapter Three: Genetic Linkage Mapping of DFNA20 to a Telomeric Region of Chromosome 17, Defined by Two Microsatellite Markers of D17S914 and D17S668

A. Introduction

Using a battery of genetic markers constructed by Research Genetics, we were able to demonstrate that the MSUDF1 family's hearing loss gene did not map to any of the known hearing loss loci (DFNAs and DFNBs) listed on the Hereditary Hearing Loss Web (http://hgins.uia.ac) (Van Camp and Smith, 1997a). A genome-wide screen for linkage was therefore undertaken using the Weber version 8Amaker panel (Research Genetics) and an ABI 377 DNA sequencer (PE Applied Biosystems). Linkage analyses were conducted with the FASTLINK version of the LINKAGE program, (Lathrop and Lalouel, 1984; Schaffer, 1996). This chapter reports the localization of DFNA20 locus to a locus on 17q25 by whole genome scan, and definition of the locus by two microsatellite markers D17S914 and D17S668 using haplotype analysis.

Three other hearing loss families with either similar hearing loss pattern or overlapped locus were later genotyped to determine whether DFNA20 was likely to be their causative gene(s).

B. Results

1. Linkage Analysis in the DFNA20 family

In this family, Positive linkage to markers at 17q25 was found with a maximum two point LOD score of 6.62 to marker D17S784 meaning at the likelihood of linkage of the causative gene to the marker is 10^6 times that the likelihood of non-linkage (Table 2A & 2B).

Haplotype analysis using additional markers in the 17q25 revealed meiotic breakpoints in individuals 409 and 421, limiting the linked region to the 12 cM between D17S1806 and D17S668 (Figure 7 & 8). Another meiotic recombination was evident in the haplotype of individual 511. This 21-year-old female has inherited the linked haplotype through marker D17S914, and showed a recombinant haplotype for markers D17S928 and D17S668. Her audiogram showed hearing within normal limits compared with the affected individual 507 at age 21 and 23. So the edge markers were determined to be D17S914 and D17S668 (Figures 5 & 6).

The obligate DFNA20 interval therefore spans approximately 5.5 cM defined by D17S914 and D17S668. This region is close to the telomere and because of increased recombination at the telomere, the physical distance is overestimated by the usual conversion assuming 1 cM ≈1 Mb. The DFNA20 interval spans only 100.1 to 133.7 $cR_{10,000}$ on the radiation hybrid (RH) map, and this corresponds to ~ 2.5 megabases, assuming 1 $cR_{10,000} \approx 25$ kbp (Figure 8).

Table 2A. Two-point LOD scores calculated in FASTLINK assuming late onset, progressive hearing loss segregating in MSUDF1 family.

	LOD Score at θ =				
Marker	0.0	0.01	0.05	0.10	cM
D17S802	4.15	4.18	4.08	3.76	106.80
D17S836	1.58	1.72	1.98	2.03	112.92
D17S1806	1.14	1.29	1.60	1.71	114.41
D17S784	6.62	6.52	6.09	5.52	116.86
D17S914	1.16	1.15	1.09	1.00	121.14
D17S668	0.30	1.28	1.75	1.77	126.46
D17S928	2.48	3.42	3.76	3.63	126.46

Family members were assigned to one of three liability classes defined in 3B, N = normal allele, D = disease allele.

Table 2B. Penetrance of Deafness for Genotype

Liability	Penetrance of Deafness for Genotype:			Age
Class	NN	ND	DD	Range
1	0.01%	1%	80%	<10 yrs
2	0.01%	43%	80%	10-23
3	0.1%	95%	99%	>23 yrs

Penetrance estimates for liability classes 1 and 3 are arbitrary and chosen to give conservative evidence for linkage. The penetrance estimate for class 2 was derived from the observation of 3 affected phenotypes out of an expected 7 in this age group (total of 14 individuals in the pedigree).

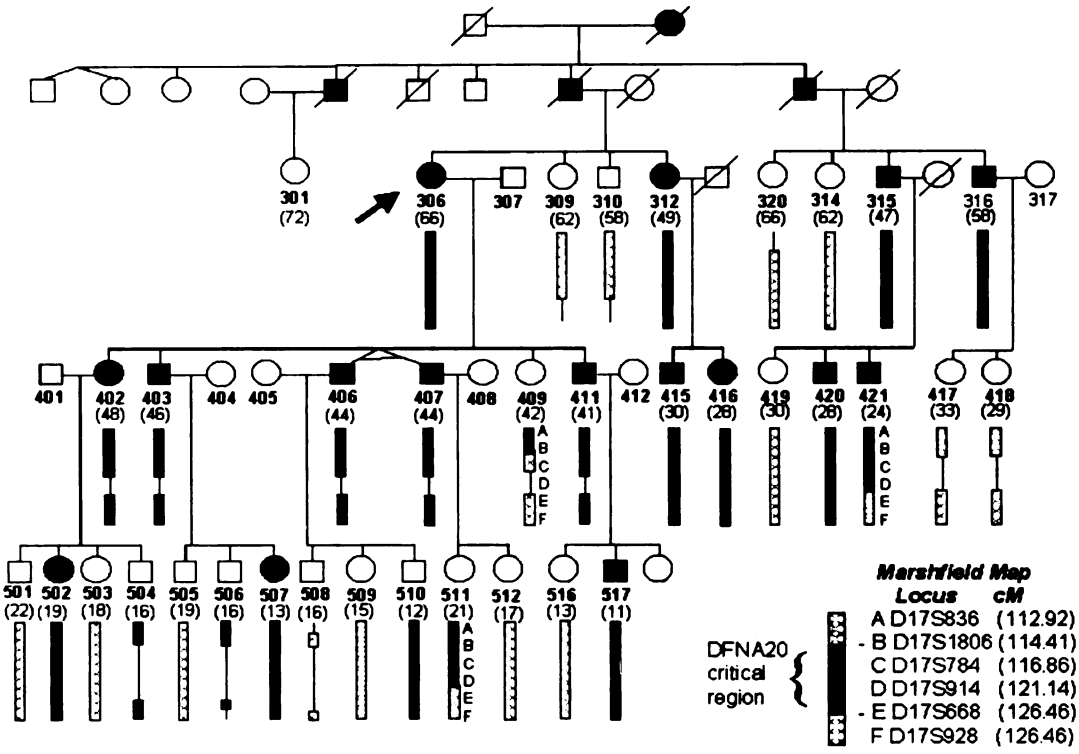

Figure 7. MSUDF1 pedigree with the DFNA20 locus defined. The bars below the pedigree symbols illustrate the segregation of chromosome 17 markers. Only the chromosome transmitted from the affected parent is pictured. Solid gray regions indicate the inheritance of the linked haplotype, light crosshatched regions indicate the unlinked haplotype, and lines indicate regions where the haplotype origin is ambiguous. Numbers below the pedigree symbols indicate those individuals from whom we obtained DNA samples and who were included in the genetic analyses. Numbers in parentheses indicate the age in years at which the audiological phenotype was determined. The genetic linkage markers and their relative positions in centi-Morgans on the Marshfield map are shown at the lower right. Recombinations defining the DFNA20 critical region are illustrated for individuals 409 and 421. A recombination is also represented by individual 511. As person 511's phenotype remains normal at age of 23, then the DFNA20 region is reduced to the interval between D17S914 and D17S668 - indicated by the black solid box in the haplotype legend in the bottom right. Because the inheritance pattern is dominant each affected individual is expected to have one mutated allele and one normal allele at the putative gene locus.

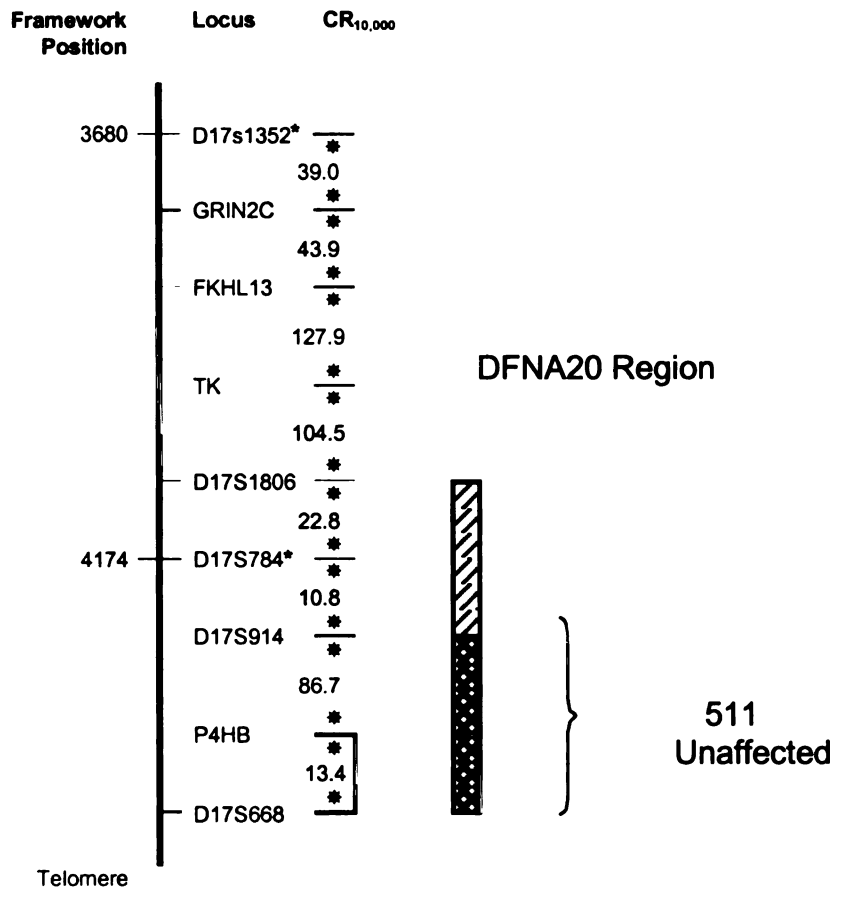

Figure 8. Framework of DFNA20 interval on RH map (http://www-shgc.stanford.edu/Mapping/) (McCarthy, 1996).

2. Linkage analysis in Boys Town 1681 family and DFNA26 families

Three microsatellite markers (kf768/kf769, kf770/kf771, and kf772/kf773) from the inside BACs, 28G8, 313F15 and 810O4, respectively, were used to do linkage analysis and decide whether the Boys Town 1681 family shares the same locus with the DFNA20 family. The haplotype around the defined DFNA20 locus is different in the affected family, nor members. There is no shared haplotype either with MSUDF1 family. We therefore conclude that the gene responsible for hearing loss in this Boys Town 1681 family does not lie in the same locus as MSUDF1 family.

The two DFNA26 families occupy a larger interval on 17q25 that overlapped with the DFNA20 family (table 3). One of the two families has the same haplotype as DFNA20 segregating with the hearing loss. It may have the same gene mutation responsible for the hearing loss as MSUDF1 family. We will use it for further confirmation of disease gene discovery as we find any meaningful gene mutation.

C. Discussion

The locus for the hearing loss gene in MSUDF1 family is designated as DFNA20. Our observations support the linkage of DFNA20 to the telomeric region of chromosome 17,

Table 3. Comparison of the haplotype of three DFNA20/26 families (kindly provided by Smith research group)

	DFNA26 Family A	DFNA26 Family B	DFNA20 Family
D17S836	**2** 2	**2** 1	**2** 3
D17S1806	**2** 3	1 3	**2** 1
D17S914	**2** 3	5 1	**2** 4
AC023494	**2** 3	**2** 1	**2** 2
AC061991	**2** 3	1 4	**2** 2
D17S928	**5** 5	3 5	**5** 4
D17S668	**1** 3	4 3	**3** 2

17q25. The DFNA20 critical interval is about 5.5 cM defined by STRs D17S914 and D17S668 with an approximate physical distance of 2.5 megabases. This region of Chromosome 17 has not been studied extensively making it one of the least well defined regions of the Human Geneome Project (http://www.ncbi.nlm.nih.gov).

Hearing loss in the two DFNA26 families maps to an extensive region that overlaps with the DFNA20 locus on 17q25. The phenotype in these families could be caused by a mutation in a different gene(s) or different or the same mutation(s) in the DFNA20. Any potential mutation(s) in a candidate gene found in DFNA20 family will therefore be tested in the two DFNA26 families.

The gene for the hearing loss in family 1681 does not appear to be in the DFNA20 locus, and was therefore excluded from further investigation by our group.

Chapter Four: Exclusion of Jackson-shaker (js) Mouse as Mouse Model for DFNA20 Gene(s)

A. Introduction

The mouse and human auditory system have many similarities, suggesting similar genetic involvement in the development of the ear in the two species. Mouse models of hearing loss have been invaluable in locating and verifying genes involved in human deafness (Probst and Camper, 1999). The cloning and identification of hearing loss genes involved in a number of forms of deafness have been achieved by combining human and mouse mapping and candidate gene evaluation. Many hearing-impaired mouse mutants are available (http://www.jax.org/research/hhim/documents/models.html). In addition, mouse models facilitate studies of the expression of genes involved in the hearing process (Steel & Brown, 1994.)

This chapter reports the exclusion of js mouse and the js-like mouse (a new mutant mouse) as mouse models for DFNA20. When a mouse gene involved in deafness has been identified, it is relatively easy to find the human homologue and to look for mutations in the DNA of deaf people (Steel and Brown, 1994.).

The locus of jackson shaker (js) mouse was mapped to mouse chromosome 11 in a region that has homology of synteny to 17q25. The "new-mutant" is thought to be allelic since it displays a similar pathology (see chapter one).

B. Results

The human cDNA homologue of the putative gene for js mouse was obtained from the Kikkawa group in Japan (Kikkawa, et al. 1998). Primers were designed for RH mapping and library screening. Two BAC clones, 420L20 and 206C8 were identified by screening a BAC library from Research Genetics, and their ends were sequenced. Meanwhile RH mapping using a combination of available G3 and TNG4 data by Dr. Robert Morell at NIDCD placed js and all other js-linked genes between D17S1352 and D17S785. This region is centromeric to the TK gene at about 104 cM while the DFNA20 gene is telomeric of TK at 117-128 cM. Therefore, the human homolog of js was excluded as a potential candidate for DFNA20.

C. Discussion

The mouse mutation, jackson-shaker (js), which causes deafness and circling behavior, maps to a region of mouse chromosome 11 that has homology with 17q25. While the homology between mouse chromosome 11 and human chromosome 17 is not well defined, it seemed possible that js was the mouse homologue of DFNA20. Our fine-map indicates js is not the mouse model for DFNA20.

It must be acknowledged that mice do not always provide perfect models for hearing genes even if a mouse homology of DFNA20 was found. Rodent retinal rods do not have myo7a expression so while mice with myo7a mutations are deaf and have vestibular

disfunction they would not be expected to show the vision problems seen in Usher B1 (El-Amraoui, et al. 1996). The most common mutation causing recessive non-syndromic deafness in humans generates a stop codon early in the coding sequence of connexin 26 (Kelsell, et al 1997). Cx26 null mice die at embryonic day 11 (Steel, et al. 1999). Although connexin 26 is expressed in many tissues in both human and mice, humans apparently have compensatory mechanisms not found in mice.

At present, there are no other candidate mouse models for DFNA20 except js and the new mutant mice, which are excluded. It will be important to follow the progress of mouse genomics in case other possible mouse models are discovered.

Chapter Five: Initial Analysis of the Possible Known Candidate Genes in the Region between D17S914 and D17S668

A. Introduction

By 1999, a number of genes were reported to lie within the 17q25 region. Table 4 lists possible candidates based on their reported 17q25 location at that time. Most of them had been mapped via human-hamster hybrid cell lines or by in situ hybridization. Comparatively few linkage markers and ESTs had been mapped to this region using radiation hybrid (RH) panels (Deloukas, et al., 1998), because of the presence of the thymidine kinase (TK1) locus at 17q25. TK1 is the selectable marker used in the construction of the commonly used RH panels and is retained in all hybrids. The retention rate for loci at 17q25, therefore, is influenced by their proximity to the TK1 locus, and this parameter must be accounted for in the RH mapping analysis. We downloaded the RH mapping data for the markers D17S1352, D17S785, D17S836 and D17S784 from the SHGC web site (http://www-shgc.stanford.edu/), and used these framework markers and the capabilities of the RHMap program (Lunetta et al., 1996) to condition the data on a selectable marker, thus allowing for unequal retention frequencies among loci. We resolved the physical order of candidate genes relative to the genetic linkage markers defining the DFNA20 interval via the Stanford G3 and TNG4 radiation hybrid panels.

We sequenced those genes that mapped within the interval, or those genes with ambiguous map location, to find out if they are the causative gene(s).

Table 4. Candidate genes on chromosome 17q25 based on data available in 1999

Gene considered and excluded by additional studies in our group	
Gene Name	**References**
Calcium channel subunit beta 1 (CNCNG)	Green, et al., 1996
Osteonectin (SPARC)	Hampton, et al., 1999
Thyroid hormone receptors (ERBA2L)	Forrest, et al. 1996a
Glutamate receptor (GRIN2C)	Takano, et al. 1993
Forkhead (FKHL 13)	Hulander, et al., 1998; Murphy, et al., 1997
Cytoplasmic actin (ACTGI)	Ueyama, et al., 1996; Skvorak, et al., 1999
Rho GDP dissociation inhibitor alpha (ARHGDIA)	Wagner, et al., 1997
Prolyl 4-hydroxylase (P4HB)	Deloukas, et al., 1998, Foster, et al., 1996
Dynein axonemal heavy chain (DNEL1)	Milisav, et al., 1996. Milisav, et al., 1998
Galanin receptor (GALR2)	Fathi, et al., 1998

B. Results

1. Candidate gene evaluation:

Listed in table 4 are genes in the chromosome region that could be candidates for DFNA20. These are CNCNG (Calcium Channel Subunit beta 1) (Green, et al., 1996); SPARC (osteonectin) (International RH Mapping Consortium and National Center for Biotechnology Information, 1999); ERBA2L (v-erb-a avian erythroblastic leukemia viral oncogene homologue 2-likez) (Gosden, et al., 1986); GRIN2C, a glutamate receptor whose mouse homologue is expressed in the outer hair cells of the cochlea (R. Wenthold, 1999, Bethesda, pers. comm.); FKHL13, whose paralogue, Fkh10, is necessary for inner ear development in mice (Hulander, et al., 1998; Murphy, et al., 1997); ACTG1 (actin, gamma 1) (Ueyama, et al., 1996); ARHGDIA (rho GDP-dissociation inhibitor α) (Wagner, et al., 1997); P4HB (the beta subunit of prolyl 4-hydroxylase) (Deloukas, et al., 1998; Foster, et al., 1996; Schuler, et al., 1996); DNEL1 (human dynein-related gene) (Milisav, et al., 1996. Milisav and Affara, et al., 1998.); GALR2 (galanin receptor type 2) (Fathi, et al., 1998); Of these, ACTG1, P4HB, and SPARC have been found in human fetal cochlear cDNA libraries (Skvorak, et al., 1999; Hampton, et al., 1999).

CNCNG is a calcium channel gene that was mapped by in situ hybridization to 17q24. A great deal of evidence demonstrates that calcium channels are important in auditory and vestibular sensory transduction (Roberts, et al., 1990. Fuchs, and Murrow, 1992. Zidanic,

and Fuchs, et al., 1995. Green, et al., 1996). In order to finemap this gene location on chromosome 17, we used a primer pair for exon 13 (cncnlb.ex13b) of CNCNG to amplify the Stanford G3 radiation hybrid panel. Results from two-point maximum likelihood analysis showed exclusion of the gene CNCNG from the DFNA20 region of 17q25.

SPARC, or osteonectin, is a calcium-binding glycoprotein expressed in developing bone. It is abundantly expressed in the inner ear, comprising 0.8% of the clones in a subtracted and normalized cochlear library (Hampton, et al., 1999). SPARC had already been mapped to 17q25 using radiation hybrid panels but the chromosomal location of SPARC is ambiguous. It has been mapped to both chromosomes 5 and 17 in the Genebridge 4 radiation hybrid panel, and to chromosome 5 by in situ hybridization (Deloukas, et al. 1998, Le Beau, et al. 1993, Schuler, et al. 1996). This suggested the possibility that a gene closely related to SPARC might be at 17q25, in which case the question of which "SPARC" gene is expressed in the inner ear needed to be resolved. The PCR primers used to place SPARC at 17q25 are from an EST sequence (Accession No. AA037365) that has been assembled into a Unigene contig for SPARC (Hs.111779). This EST shows no sequence similarity to the full-length cDNA (Genbank Accession No. J03040) or to the twenty ESTs from the Morton fetal cochlear library that shows similarity to SPARC. Its assembly into Unigene Hs.11179 is most likely due to the presence of a MER3 repetitive element. Thus, we consider the association of AA037365 with SPARC to be is spurious, and no SPARC-like gene is in the DFNA20 region.

ERBA2L, or ERBA2-like, also mapped to the region using radiation hybrid panels. This gene would be a promising candidate on the basis of the known significance of thyroid hormone receptors (originally designated "Erba"s) in cochlear pathologies. Thyroid hormone metabolism is an important constituent of cochlear development and function (Forrest, et al. 1996a, Forrest, et al. 1996b, Forrest, et al. 1996c, Rusch, et al. 1998). Mice, deficient for the thyroid hormone receptor beta gene (Thrb $^{-/-}$), have impaired auditory function with no other obvious neurological defects (Forrest, et al. 1996a). The human orthologue, THRB or ERBA2, was originally mapped to 17q11-q21 on the basis of in situ hybridization data (Gosden, et al. 1986). An additional weak hybridization signal was detected at 17q25, which presumably was the origin of the "ERBA2L" locus designation. The THRB locus was subsequently mapped to 3p24.3, and the THRA (thyroid hormone receptor alpha) gene, which has sequence similar to THRB, was placed at 17q11.2 (Drabkin, et al. 1988). There is no Genbank entry for ERBA2L, and no clones for this gene have been published. The GDB entry for ERBA2L lists PCR primers that amplify a 285 bp product and were used to map ERBA2L relative to P4HB using a high resolution RH panel (Foster, et al. 1996). A BLAST search of these primers reveals that they actually complement P4HB, and are in the correct orientation to amplify a 285 bp product from exon 11 of P4HB. Thus the ERBA2L locus does not exist as such, and the original designation probably arose due to modest sequence similarity between ERBA2 in situ probes and P4HB.

We mapped the remaining candidate genes relative to the genetic linkage markers defining the DFNA20 interval via the Stanford G3 radiation hybrid panel. We

downloaded the RH mapping data for the markers D17S1352, D17S785, D17S836 and D17S784 from the SHGC web site (http://www-shgc.stanford.edu/), and used these framework markers and their defined orders to initiate our own map. The best-ordered maps for markers centromeric to TK1 are shown in Table 5.

GRIN2C and FKHL13 could be excluded unambiguously from the DFNA20 interval based on these results. ACTG1 was also excluded from the DFNA20 interval. ARHGDIA and P4HB are distal to D17S668 in the DFNA20 interval. DNEL1 and GALR2 were ambiguous on the map.

P4HB has been shown to be identical in amino acid sequence to the cellular thyroid hormone-binding protein p55 (Popescu, et al., 1988) and was found in the human fetal cochlear cDNA library (Skvorak, et al., 1999). We designed PCR primers to amplify all 11 exons of P4HB from genomic DNA from several affected and unaffected family members of the DFNA20 family. The PCR product included all exon/intron boundaries.

Sequencing of the amplicon showed a c to t transition at position -4 relative to the exon9/intron9 splice site. The resulting sequence surrounding the donor site of exon 9 is AGGACGgtgtgccttccc. This change blocks a Hha I site (GCGC). We used the HhaI to digest PCR products in order to determine if this change cosegregates with the DFNA20 phenotype. The affected individuals are either heterozygous or homozygous for the t at position 1971(using the Genbank numbering); several unaffected individuals are

heterozygous, including at least three instances of individuals who married into the family (Figure 9 & table 6).

We also typed 8 random Northern European individuals for their genotypea at position 1971 using the human diversity DNA samples (Coriell Cell Repositories) and found 5 heterozygous c/t, 2 homozygous t/t and one homozygous c/c. Thus this sequence variant appears to be a common polymorphism and not a significant splice-site mutation. Sequencing of P4HB suggests that it is not responsible for the DFNA20 phenotype.

Dyneins are molecular motors that form large multi-subunit protein complexes. Cytoplasmic dyneins are believed to be involved in the motility of a variety of intracellular components such as endocytotic vesicles or condensed chromosomes during mitosis (Holzbaur, et al., 1994). Axonemal dyneins are involved in the movement of cilia and flagella (Witman, 1992). DNEL1 is a human dynein-related gene. It is expressed specifically in testis and shares a high degree of sequence identity and amino acid similarity with the C-terminal region of the outer arm axonemal dynein β–heavy chains derived from sea urchin and other species (Milisav, et al., 1996). DNEL1 has previously been mapped to the DFNA20 interval (Milisav, et al., 1996. Milisav, et al., 1998) but ambiguously mapped in our two RH panels. Based on the significance of molecular motor in the hearing process the DNEL1 was considered as strong candidate for DFNA20.

The full-length cDNA of DNEL1, X99947, is 3.163 kb. The potential DNEL1 coding exons on BACs (AC005209 & AC005410) were predicted. All 15 exons of DNEL1 were

sequenced. No significant changes were found. More importantly, DNEL1 was subsequently excluded from the DFNA20 region on geneMap'99 (http://www.ncbi.nlm.nih.gov/genemap/) even though it had been mapped to the DFNA20 region by Milisav's group in 1998.

GALR2 was mapped to17q25 (Fathi, et al., 1998). Sequencing of GALR2 included the exon/intron boundaries. Results gave no significant nucleotide change. ARHGDIA was sequenced by my lab colleague, and again no significant variation was found.

Table 5. Locus order of markers and genes determined by radiation hybrid mapping.

Group	Likelihood ratio	Locus Order
1	1.0	D17S1352* – GRIN2C – FKHL13 – D17S785* – TK1 – D17S836* – D17S784* – ACTG1
	1.3	D17S1352* – GRIN2C – FKHL13 – D17S785* – TK1 – ACTG1 – D17S836* – D17S784*
2	1.0	ACTG1 – TK1 – D17S836* – **D17S1806** – D17S784* – **D17S914** – **D17S668** – P4HB – ARHGDIA
	14.9	TK1 – ACTG1 – D17S836* – **D17S1806** – D17S784* – **D17S914** – **D17S668** – P4HB – ARHGDIA
	41.9	ACTG1 – TK1 – D17S836* – **D17S1806** – D17S784* – **D17S914** – P4HB – ARHGDIA – **D17S668**
	106.0	TK1 – ACTG1 – D17S836* – **D17S1806** – D17S784* – **D17S914** – P4HB – ARHGDIA – **D17S668**

Locus orders are based on G3 RH panel (for Group 1) and a combination of the G3 and TNG4 RH panels (for Group 2). The order for framework markers (asterisks) relative to the TK1 locus was formed, others were allowed to vary. The markers flanking the DFNA20 interval are in bold font. D17S914 would become the distal flanking marker because person 511 retains a normal hearing phenotype as she ages.

Table 6. Summary of the genotype result for the SNP on exon 9 of P4HB in MSUDF1 family

Family ID	Genotype	Family ID	Genotype	Family ID	Genotype
301	+/+	402	-/-	419	+/-
306	-/-	403	-/-	420	+/-
307	+/-	404	+/+	421	+/-
309	+/-	406	-/-	502	+/-
310	+/+	407	-/-	505	+/-
312	+/-	408	+/+	506	+/-
314	+/-	409	+/-	507	+/-
315	-/-	411	+/-	511	+/-
316	-/-	415	-/-		
401	+/+	418	+/-		

Note: + represents uncut allele, - represents cut allele (refer to figure 9).

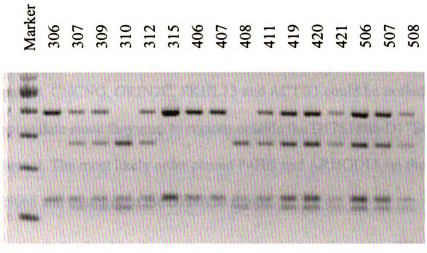

Figure 9. SNP analysis of C to T transition in P4HB at –4 relative to the exon 9/intron 9 splice site. The transition abolishes a Hha1 site (GCGC) in the amplicon produced P4HB specific primers. Digestion shows four bands (507bp, 260bp, 130bp, and 112bp) if it is heterozygous, three bands (112bp, 130bp, 507bp) if homozygous uncut. Normal homozygous cut produces three bands (112bp, 130bp, and 260bp).

B. Discussion

In the absence of a mouse model, we adopted a candidate gene approach. We mapped the genes CNCNG, GRIN2C, FKHL13, ACTG1, P4HB and ARHGDIA, relative to the markers defining the DFNA20 region on a radiation hybrid map using the Stanford G3 and TNG4 panels. CNCNG, GRIN2C, FKHL13 and ACTG1 could be excluded based on the RH mapping data since they map to regions outside the D17S1806-D17S668 DFNA20 interval. The most likely order placed P4HB and ARHGDIA on the proximal side of D17S668 and thus within the DFNA20 region.

In mammals, galanin mediates a diverse spectrum of biological activities in both central and peripheral nervous systems as well as in the endocrine system by interacting with high-affinity cell surface receptors. GALR2 is such a human galanin receptor localized to chromosome 17q25.3 (Fathi, et al., 1998). The two coding exons of the human GALR2 gene were sequenced although GALR2 has an ambiguous map position within DFNA20 interval.

Sequencing of P4HB, DNEL1, GALR2 and ARHGDIA suggests that none is the DFNA20 gene. However, we only sequenced the exons and exon/intron boundary regions and the regulatory regions need to be explored further.

Chapter Six: Refinement of the DFNA20 Critical Region and Construction of a Partial Physical Contig across It

A. Introduction

Chapter three describes a genome-wide scan and RH map limited DFNA20 interval a genetic region of 5.5 cM, approximately 2.5 megabases assuming 1 $cR_{10,000} \approx 25$ kbp. Chapters four and five exclude the js mouse model that mapped to the DFNA20 homologous region and a number of potential candidate genes on 17q25 with known functions related to the hearing process.

In order to reduce the number of the genes to be characterized, it was necessary to narrow the DFNA20 interval as much as possible, therefore more family members and more informative markers, microsatellites or SNPs, were needed. In the absence of known genes to be analyzed, genomic sequences could be used for predicting potential genes and looking for the full-length transcripts, so a physical contig was needed. This chapter reports the refinement of the DFNA20 interval and the construction of a partial physical contig across it using microsatellites and SNPs.

Results

1. Markers and construction of the physical contig across DFNA20 critical interval

Potential microsatellite markers were identified in sequenced BACs by examining for di, tri , or tetra nucleotide repeats if the BACs are reported on 17q25. Primer pairs (see material and methods) for microsatellite markers were developed from sequenced BACs 494E11, 31B8, 11J14, 810O4, 28G98, 313F15, 498C9, 497H17, 450A4 which were typed for linkage in the MSUDF1 family, a typical genotyping result from BAC 497H17 is shown in Figure 10.

These data allowed us to put BACs: 494E11, 417L17, 442P22, 31B8, 455O6, 11J14 149I9, 163N19, 2544B16, 810O4, 28G8, 313F15 inside of the interval, which is linked to the DFNA20 phenotype. The results of the genotyping excluded BACs: 334C17, 353N14, 98G11, 498C9, 497H17, 450A4 to outside the interval (Figure 11 & Table 8).

SNP testing was also attempted to place EST's mapped to the interval if no sequenced BACs were found. Primer pairs (kf697/kf698) designed from ESTs sequences of stSG 42707 were used to amplify a region (GC-rich) where a BstN1 polymorphism is generated. In the same way, primer pairs (Kf813/814, Kf815/816, Kf897/898) from ESTs clusters of stSG52095, stSG46575 and stSG55037 were used to amplify regions where a MboI (DpnII), AluI and BsmBI sites were generated, respectively. The results show that they are not informative in the MSUDF1 family.

STS sequences placed in the region on the RH map were ordered using the BAC information. The genotyping results (table 7) showed that 38 STSs were out of the DFNA20 interval, 16 STSs were inside the DFNA20 interval, and the other 14 STSs were

on the BAC 2654F23, and 2116F7, 2283E7, CTD2625A2 or not on any BACs, respectively, and are possibly in the DFNA20 interval. Marker D17S914, which originally defined the centrometric boundary of the interval was not found in any of the sequenced BACs, therefore, 353N14 and 98G11 were used to search GSS database, unsequenced BACs: 2529O21, 3106O6, 3108O8, 2235I11, 2011A7, 2116F7 and 2283E7 were identified. The PCR results using a primer pair for D17S914 shows that D17S914 hits 2529O21, 3106O6, and 3106O8.

2. BAC library screening

StSG52023, stSG22534, stSG29570 –stSG53539stSG4925, sts-AA034065, A006A06 were used to screen the BAC library from Research Genetics (see chapter one background). BACs 442P22, 290H12, 494P19/20, 370H10, 290H10, 314M5 and 265K21 are identified by PCR using STS primer pairs (table 8). The end sequences are obtained and used to search the HGP and Celera database. However, no other sequenced BACs except 442P22 searched out 31B8 and 417L15, which were used to extend the contig towards the centromere (table 8, figure 11).

Figure 10. Microsatellite (CA repeat) found in 497H17 is typed in MSUDF1 family. The arrow shows the phenotype-linked allele. The unaffected individual 511has this allele, affected individuals either inherit this allele (420, 406, 407) or not (421), so 497H17 is recombined out of the DFNA20 interval. This is just an example for my linkage analysis to decide which BACs are linked to hearing loss, which BACs are not.

Table 7. Fine map of STSs on 17q25

STSs inside the interval	STSs ambiguous map	STSs outside the interval
stSG52023, stSG22534, stSG29570, stSG26434 stSG46575, sts-T49250, stSG52095, stSG9053, stSG26104, stSG53536, stSG53539, A006D02, stSG4739, A009F31, AA05R46, stSG30704	stSG25763, sts-N27028, sts-X62025, stSG53899 stSG42707, stSG63438 stSG9619, stSG22051 stSG3097, A002B14 A001Z20, stSG55037 StSG28692, N27169	stSG1293, stSG48048, A005O39, stSG41701, stSG22467, stSG52802, stSG3511, stSG62461, stSG31449, stSG41670, stSG38967, stSG51680, stSG48230, stSG28519, stSG63438, stSG26127, sts-AA034065, sts-AA036786, stSG414454, stSG41935, stSG4925, A006A06, stSG50025, sts-R37666, stSG9499, U32183, A005O08, stSG1600, stSG312, stSG46767, stSG4234, stSG1475, stSG52829, D29040, stSG48549, stSG46959, stSG53499, stSG52348

Table 8. RH map, STSs marker, BACs from HGP and genomic Scaffold from Celera.

RH	STSs	Searched BACs	Unique Seq.	Primer Kf #	Screened BACs	Celera Scaffold
521.27	stSG12593	334C17				
521.27	stSG48408	334C17				GA_x54KRCCRRV5(495.600kb)
521.47	A005O39	334C17				GA_x54KRCCRRV5(495.600kb)
521.47	stSG41701	334C17				GA_x54KRCCRRV5(495.600kb)
521.47	**D17S784**	353N14		Kf824/825		GA_x54KRCCRRV5(495.600kb)
521.47	stSG22467	353N14/98G11		Kf1164/1165		GA_x54KRCCRRV5(495.600kb)
521.47	stSG52802	353N14/98G11		Kf1056/1057		GA_x54KRCCRRV5(495.600kb)
521.47	stSG3511	353N14/98G11		Kf1052/1053		GA_x54KRCCRRV5(495.600kb)
521.47	stSG62461	353N14/98G11		Kf1048/1049		GA_x54KRCCRRV5(495.600kb)
521.47	stSG31449	353N14/98G11		Kf10501051		GA_x54KRCCRRV5(495.600kb)
521.47	stSG41670	353N14/98G11		Kf1054/1055		GA_x54KRCCRRV5(495.600kb)
521.77	stSG28519	353N14/2116F7		Kf1168/1169		GA_x54KRCCRRV5(495.600kb)
521.77	stSG38967	353N14		Kf1166/1167		GA_x54KRCCRRV5(495.600kb)
521.77	stSG51680	353N14/2116F7		Kf1172/1173		GA_x54KRE4KEJ8 (0.807kb) U
521.77	stSG48230	353N14/2116F7		Kf1170/1171		GA_x2HTBL5BKK5 (1.650kb) U
521.47	stSG63438	2283E7/2116F7		Kf1107/1108		
521.47	A001Z20		ATC17	Kf891/892		GA_x54KRCCRRV5(495.600kb)
521.47	stSG55037		ATC17	Kf889/890		GA_x54KRCCRRV5(495.600kb)
521.77	stSG28692			Kf1109/1110		GA_x2HTBL60EAK(29.126kb)
526.70	N27169			Kf1111/1112		GA x2KMHMR4VU4 (0.928 kb)
521.47	stSG9619	CTD2526A2		kf1103/1104		GA_x2HTBKTCFE2 (59.942kb)
521.47	stSG22051	CTD2526A2		Kf1105/1106		GA_x2HTBKTCFE2 (59.942kb)
521.47	stSG3097	CTD2526A2		Kf1099/1100		
521.47	A002B14	CTD2526A2		Kf1101/1102		
527.20	sts-N27028	2654f23		Kf632/633		
527.20	stSG25763	2654f23		Kf636/637		
529.92	stSG51913			Kf600/601		GA_x2HTBL4K91W (79.984kb)
527.20	stSG42707	2654f23	RPML12	Kf628/629		GA_x2HTBL4K91W (79.984kb)
527.42	stSG53899	2654f23		Kf614/615		GA_x2HTBL4K91W (79.984kb)
527.20	sts-X62025	2654f23		Kf634/635		GA_x2HTBL4K91W (79.984kb)
527.20	stSG52023	31B8	D17S613	kf899/900	442P22	GA_x2HTBKV89W7 (373.942kb)
527.78	stSG22534	31B8/428B22		Kf608/609	290H12	GA_x2HTBKV89W7 (373.942kb)

Table 8. RH map, STSs marker, BACs from HGP and genomic Scaffold from Celera.

RH	STSs	Searched BACs	Unique Seq.	Primer Kf #	Screened BACs	Celera Scaffold
521.27	stSG12593	334C17				
521.27	stSG48408	334C17				GA_x54KRCCRRV5(495.600kb)
521.47	A005O39	334C17				GA_x54KRCCRRV5(495.600kb)
521.47	stSG41701	334C17				GA_x54KRCCRRV5(495.600kb)
521.47	**D17S784**	353N14		Kf824/825		GA_x54KRCCRRV5(495.600kb)
521.47	stSG22467	353N14/98G11		Kf1164/1165		GA_x54KRCCRRV5(495.600kb)
521.47	stSG52802	353N14/98G11		Kf1056/1057		GA_x54KRCCRRV5(495.600kb)
521.47	stSG3511	353N14/98G11		Kf1052/1053		GA_x54KRCCRRV5(495.600kb)
521.47	stSG62461	353N14/98G11		Kf1048/1049		GA_x54KRCCRRV5(495.600kb)
521.47	stSG31449	353N14/98G11		Kf10501051		GA_x54KRCCRRV5(495.600kb)
521.47	stSG41670	353N14/98G11		Kf1054/1055		GA_x54KRCCRRV5(495.600kb)
521.77	stSG28519	353N14/2116F7		Kf1168/1169		GA_x54KRCCRRV5(495.600kb)
521.77	stSG38967	353N14		Kf1166/1167		GA_x54KRCCRRV5(495.600kb)
521.77	stSG51680	353N14/2116F7		Kf1172/1173		GA_x54KRE4KEJ8 (0.807kb) U
521.77	stSG48230	353N14/2116F7		Kf1170/1171		GA_x2HTBL5BKK5 (1.650kb) U
521.47	stSG63438	2283E7/2116F7		Kf1107/1108		
521.47	A001Z20		ATC17	Kf891/892		GA_x54KRCCRRV5(495.600kb)
521.47	stSG55037		ATC17	Kf889/890		GA_x54KRCCRRV5(495.600kb)
521.77	stSG28692			Kf1109/1110		GA_x2HTBL60EAK(29.126kb)
526.70	N27169			Kf1111/1112		GA x2KMHMR4VU4 (0.928 kb)
521.47	stSG9619	CTD2526A2		kf1103/1104		GA_x2HTBKTCFE2 (59.942kb)
521.47	stSG22051	CTD2526A2		Kf1105/1106		GA_x2HTBKTCFE2 (59.942kb)
521.47	stSG3097	CTD2526A2		Kf1099/1100		
521.47	A002B14	CTD2526A2		Kf1101/1102		
527.20	sts-N27028	2654f23		Kf632/633		
527.20	stSG25763	2654f23		Kf636/637		
529.92	stSG51913			Kf600/601		GA_x2HTBL4K91W (79.984kb)
527.20	stSG42707	2654f23	RPML12	Kf628/629		GA_x2HTBL4K91W (79.984kb)
527.42	stSG53899	2654f23		Kf614/615		GA_x2HTBL4K91W (79.984kb)
527.20	sts-X62025	2654f23		Kf634/635		GA_x2HTBL4K91W (79.984kb)
527.20	stSG52023	31B8	D17S613	kf899/900	442P22	GA_x2HTBKV89W7 (373.942kb)
527.78	stSG22534	31B8/428B22		Kf608/609	290H12	GA_x2HTBKV89W7 (373.942kb)

527.78	stSG29570	31B8	D17S1168	kf604/605	290H12	GA_x2HTBKV89W7 (373.942kb)
527.20	StSG26434	455O6	D17S761	kf574/575		GA_x2HTBKV89W7 (373.942kb)
531.55	Sts-T49250	455O6/11j14		Kf630/631		GA_x2HTBKV89W7 (373.942kb)
528.61	stSG52095	455O6/11j14		Kf602/603		GA_x2HTBKV89W7 (373.942kb)
527.20	stSG46575	455O6/11j14		Kf626/627		GA_x2HTBKV89W7 (373.942kb)
527.78	stSG9053	455O6/11j14		Kf612/613		GA_x2HTBKV89W7 (373.942kb)
517.05	StSG26104	455O6/11j14	RPS29	kf1046/1047		GA_x2HTBKV89W7 (373.942kb)
527.42	stSG53536	2544b16		Kf622/623		GA_x54KRCDA952 (445.106kb)
527.42	stSG53595	2544b16		Kf624/625	494P19/20	
527.42	A006D02	2544b16		Kf620/621		
527.78	stSG4739	2644b16		Kf610/611		
527.78	A009F31	810O4/2544b16		kf606/607		GA_x54KRCDA952 (445.106kb)
527.42	A005R46	810O4/2544b16		kf618/619		GA_x54KRCDA952 (445.106kb)
521.47	stSG30704	313F15		Kf873/874		GA_x54KRCDA952 (445.106kb)
527.42	stSG31435			Kf616/617		GA_x2HTBKV89PU (0.797 kb)
536.52	stSG53488			Kf566/567		GA_x2HTBL4B1CR (10.823kb)
539.92	sts-AA036786	498C9				GA_x2HTBL4VER4(15.487kb)
537.15	stSG41935	498C9	PCYT2			GA x2HTBLPTDRR (203.55kb)
537.15	stSG4925	498C9	**ARHGDIA**			GA_x2HTBLNVSH8 (15.894kb)/
530.84	stSG26127	498C9	**P4HB**		370H10	GA_x2HTBL4VFBE(19.001kb)
530.84	sts-AA034065	498C9	**P4HB**		370H10/290H10	GA_x2HTBL4VFBE(19.001kb)
537.15	stSG41454	498C9			370H10	
537.15	A006A06			Kf548/549	370H10/314M5/265K21	GA_x2HTBLNVSH8 (15.894kb)/
537.15	stSG9499	650J16	RFNG	Kf554/555	370H10/314M5/265K21	GA_x2HTBKWY7A8 (67.666kb)
537.15	sts-R37666	650J16	RFNG	Kf552/553	370H10/314M5/265K21	GA_x2HTBKWY7A8 (67.666kb)
537.15	stSG50029	650J16	GPSI	Kf550/551	370H10/314M5/265K21	GA_x2HTBKWY7A8 (67.666kb)
539.46	U31285	516M14			370H17	
541.28	A005O08	516M14				
544.07	stSG1600	516M14				
501.65	StSG312	516M14	CSNK1D			
539.36	**D17S928**	**516M14**		Kf826/827		GA_x2HTBL4B1CV (96.600kb)
536.52	stSG46767	497H17				GA_x54KRCCUJ1A (500.000kb)
536.52	stSG4134	497H17				GA_x54KRCCUJ1A (500.000kb)
536.52	stSG1475	497H17/450A4				GA_x54KRCCUJ1A (500.000kb)
536.52	stSG52829	497H17/450A4				GA_x54KRCCUJ1A (500.000kb)

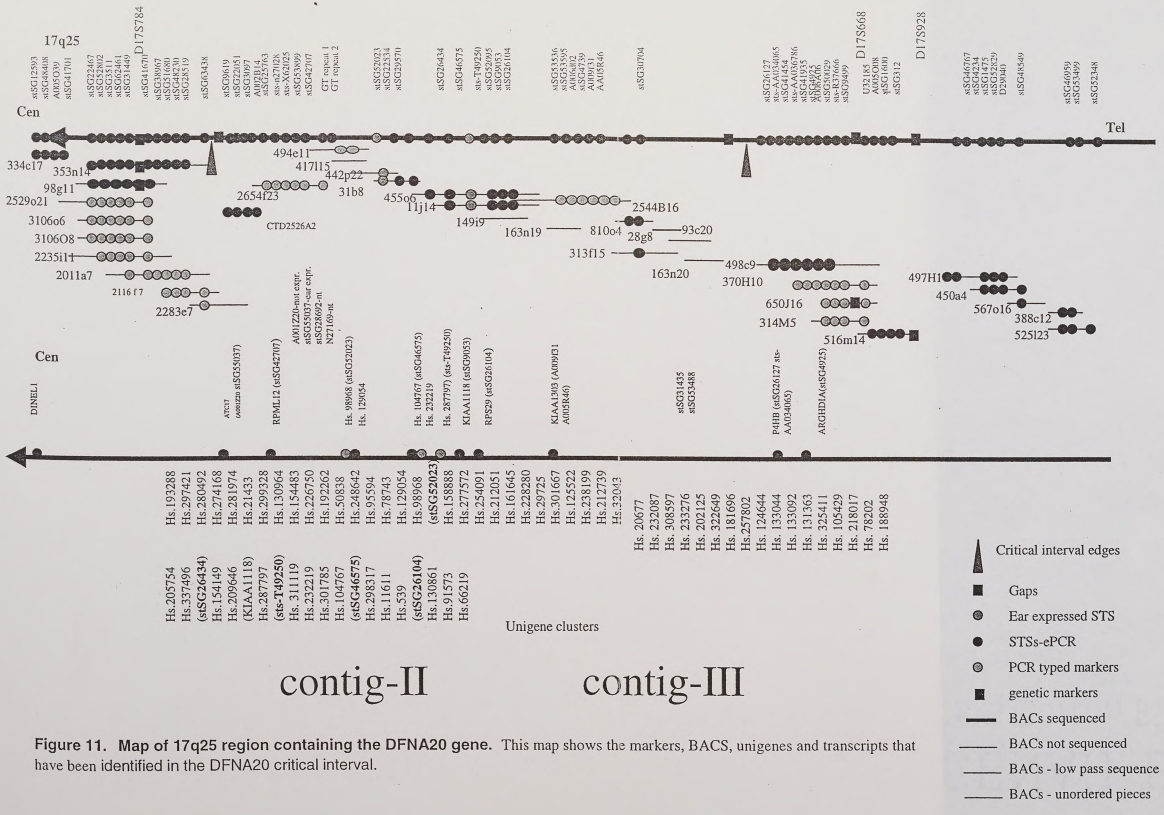

Figure 11. Map of 17q25 region containing the DFNA20 gene. This map shows the markers, BACS, unigenes and transcripts that have been identified in the DFNA20 critical interval.

C. Discussion

Microsatellites generally occur in non-coding regions of the genome and appear to be uniformly distributed. They have a wide range of uses including linkage and association studies looking for disease loci, cancer studies that look for loss of heterozygosity and population genetics. The strategy used in this study was to design primers from the sequences flanking the microsatellite, amplify the region by PCR, test for unique amplification, then type family members to look for a polymorphism. Markers found to be polymorphic were then used to look for the recombinations that would narrow the DFNA20 region.

We used radioactively labeled genotying reactions based on previously sequenced BACs to rule in and out the sequenced BACs that lay within DFNA20 critical interval.

The sequence of the human genome is one of the largest genomes to be extensively sequenced so far. Much work remains to be done to produce a complete finished sequence. The pericentromeric and subtelomeric regions of chromosomes are filled with large duplications of sequence from elsewhere in the genome, which makes the correct assembly of sequenced fragments difficult (International Human Genome Consortium. 2001). The sequence of the telomere region of chromosome 17 is still very incomplete, there are only two BAC clones (28G11 and 313F15) with finished sequence and there are numerous fragments in the rest of the BACs in the DFNA20 interval. The region of the chromosome containing the DFNA20 gene is flanked by markers D17S914 and

D17S668. The latter one is in the BAC clone 650J16 (Table 8 & Figure 11). There are no sequenced BAC clones containing D17S914, but unsequenced BACs 2529O21, 3106O6 and 3108O8 contain D17S914 based on our PCR results only. We have excluded the telomeric and centromeric contigs and confirmed that contigs II and III are in the interval by typing microsatellites found in BACs within the contigs. Contigs II and III are bridged by a partially sequenced BAC (2544B16). By searching the GSS database and by screening the RPCI-11 BAC library we have identified several more BACs that extend the contig toward the centromere and telomere. Using the BACs end sequences to search the genomic database, some BACs in this interval hit the BACs on different chromosomes instead of chromosome 17. We have some hints that there may be low-copy repeat material present in the DFNA20 interval, which causes misassignment or non-assignment of clones that are supposed to be in the region. Based on the HGP data the DFNA20 critical interval is currently limited to a physical distance of approximately 1.5 megabase. The partial physical contig covers approximately 1 megabase.

Figure 11 shows only a partial physical contig across the DFNA20 interval; the two edges have not been bridged together. More data from HGP and Celera are needed in order to finish the physical contig and minimize the physical distance of the DFNA20 critical interval.

Chapter Seven: Characterization of the Cochlear STSs or ESTs- represented Novel Genes and Analysis of the cDNA from the Chromosome Somatic Cell Hybrid

A. Introduction

In previous chapters, using a whole genome scan, I described how the locus for the causative gene was mapped to a 5.5 cM region on 17q25. The DFNA20 region is defined by microsatellite markers D17S914 and D17S668 (Morell, et al. 2000). We excluded all candidate genes that were known to map to the region as the cause of hearing loss in the DFNA20 family. The JS mouse was also ruled out as a model for this family's HL. Using additional markers and genomic databases from HGP and Celera, the regional boundaries were further refined. A partial physical contig containing about 1 megabase was then constructed (Figure 11).

Without any known candidate genes or mouse model available, the efficient way to look for the causative gene is to explore the STSs mapped in the interval. Currently 16 STSs are put inside the region; they represent a number of ESTs, which are part of different transcripts. This chapter reports the analysis of 4 interesting transcripts –newly identified by the HGP, chosen because they are either expressed in the human cochlear library or have important domain(s).

B. Result

1. Identification of interesting transcripts:

The cochlear library was provided by Dr. Robert Morell at NIDCD of NIH, this library was used to look at the cochlear expression of transcripts and decide which transcripts have the highest priority to characterize.

Total phage DNA was extracted from the human fetal cochlear cDNA library grown in LB medium with kanamycin (Israel, 1993). PCR was performed on the total fetal cochlear phage DNA to determine the cochlear expression of the STSs that are mapped within the interval.

Twenty-three STS were tested and three (stSG52023, sts-T49250, stSG55037) showed clear ear expression in this fetal cochlear library (Table 9).

2. Genotyping of Somatic Cell Hybrids

We obtained three somatic hybrid cell lines, #8, #15, #19, each containing a single human chromosome 17, constructed from the proband's lymphocytes by GMP Genetics using mouse E2 cells as recipient. Using several microsatellite markers from BACs that are

both inside and outside of the DFNA20 interval, we demonstrated that cell line #15 has a copy of the affected chromosome, cell lines #8 and #19 have a copy of the normal one (Figure 12).

3. Characterization of gene 1-----Hs. 98968 represented by stSG52023

stSG52023 was the first ear-expressed STS identified, it belonged to the unigene cluster, Hs.98968. This cluster is expressed in various types of tissues including brain, ear, kidney, lung, muscle, whole embryo, head, neck, testis, germ cell, prostate, etc. It contained 48 partial EST sequences from different I.M.AG.E cDNA clones (http://www.ncbi.nlm.nih.gov/UniGene/). Some of the web-reported ESTs are read through the 3' end of the clone, some read through the 5' end. We ordered three I.M.A.G.E cDNA clones and sequenced them; I.M.A.G.E 2168160 with 1.5kb insert from brain; I.M.A.G.E 252978 with 0.5kb from ear; I.M.A.G.E 774065 (no size information) from whole embryo. A cDNA sequence of 1.5kb with a poly (A) site was obtained, but it contained no good open reading frame.

In order to identify the actual ear expressed cDNA sequence, stSG52023 was used to screen the human fetal cochlear phage cDNA library. One phage clone was identified and sequenced, it is part of the sequence on the 3' end of the I.M.A.G.E 2168168 cDNA clone, and is 687 bp long.

Using the partial insert from IMAGE 774065 (done by Dr. Karen Friderici), multiple

tissue northern blots were done. This showed that this STS represented gene is highly

Table 9. The result of cochlear expression of the STSs that are mapped within the DFNA20 critical interval.

STSs	Unigene cluster	Primer pair in Kf #	Cochlear expression
stSG3074	Hs.226372	Kf873/874	no
A009F31	Hs.20677	Kf606/607	no
A005R46	Hs.20677	Kf618/619	no
stSG29570	Hs.154483	Kf604/605	no
stSG52023	Hs. 98968	Kf458/459|899/900	**Yes (gene 1)**
stSG22534	Hs.193288	Kf608/609	no
stSG55037	ATC17*	Kf889/890	**Yes**
A001Z20	ATC17*	Kf891/892	no
sts-T49250	Hs. 287797	Kf630/631	**Yes (gene 2)**
stSG9053	Hs. 209646	Kf612/613	No (gene 4)
stSG26434	Hs. 337496	Kf574/575	no
stSG46575	Hs.104767	Kf626/627	No (gene 2)
stSG52095	--	Kf602/603	no
stSG26104	Hs.539	Kf1046/1047	no
stSG25763	Hs.164256	Kf636/637	no
sts-N27028	Hs.43880	Kf632/633	no
sts-X62025	Hs.1857	Kf634/635	no
stSG53899	--	Kf614/615	no
stSG42707	Hs.109059 (RPML2*)	Kf628/629	no
stSG53536	--	Kf622/623	no
stSG53595	--	Kf624/625	no
A006D02	Hs.182648	Kf620/621	no
stSG4739	Hs.7936	Kf610/611	no

*ATC17 (anion transporter analog, 2.5kb) was identified by Dr. Mount, Harvard, it was sequenced by a colleague in our lab. No heterozygous changes were found.

*RPML2 (mitochondrial ribosomal protein L2, 1 kb) was identified by Dr. J. Sylvester, Miami Children Hospital. It was sequenced on the genomic basis from proband. No heterozygous changes were found.

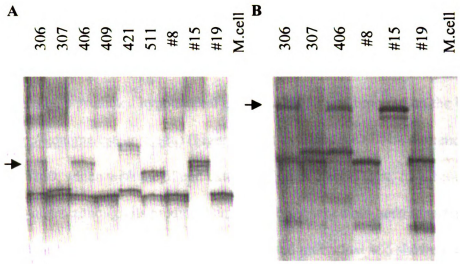

Figure 12. Genotyping of the somatic cell hybrids. **A** shows a microsatellite marker from BAC 497H17 outside the region; **B** shows a microsatellite marker from BAC 313F15 inside the region. The microsatellite markers were used to type the cell lines for comparison with the proband (306) and her son (406). Arrow indicates the affected allele, cell line #15 contains the affected chromosome.

expressed in skeletal muscle and it is approximately 1.35kb in size. Using the whole insert of about 687bp in the screened phage cDNA clone as probe, multiple tissue northern blot gives the same result. Using stSG52023 sequences (176bp purified from PCR product) as probe, multiple tissue northern blot shows that it is highly expressed in skeletal muscle and has two sizes of 1.35kb and 9.5kb in length (Figure 13).

Recently, a predicted hypothetical gene FLJ23058 was reported on the UCSC web site (http://genome/cse.ucsc.edu) (Figure 14), it overlaps the cDNA sequence of I.M.A.G.E. 2168160, which with the 5' end extension, gives 2 kb in total. Its potential function is not yet known although its predicted amino acid sequence has been reported (Figure 15). Sequences of the coding region in the affected individual 406 showed no variation.

4. Characterization of gene 2----- Hs.8961 and Hs.104767 represented by sts-T49250 and stSG46575

Originally gene 2 was considered to be two separate genes but as data from EST sequencing emerged it became clear that these were part of the same transcription unit and eventually merged into one unigene. The unigene cluster Hs. 8961 (later renamed as Hs.287797) was chosen because it has an ear-expressed STS, sts-T49250. This unigene cluster has wide tissue expression including aorta, bone, breast, colon, esophagus, eye, germ cell, heart, kidney, lung, muscle, placenta, whole embryo, etc. I.M.A.G.E 2447381

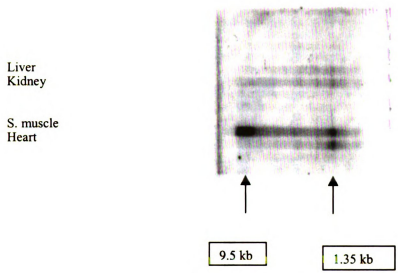

Figure 13. Northern blot hybridization using the PCR product of 176 bp long. The expressed tissues are skeletal muscle and heart.

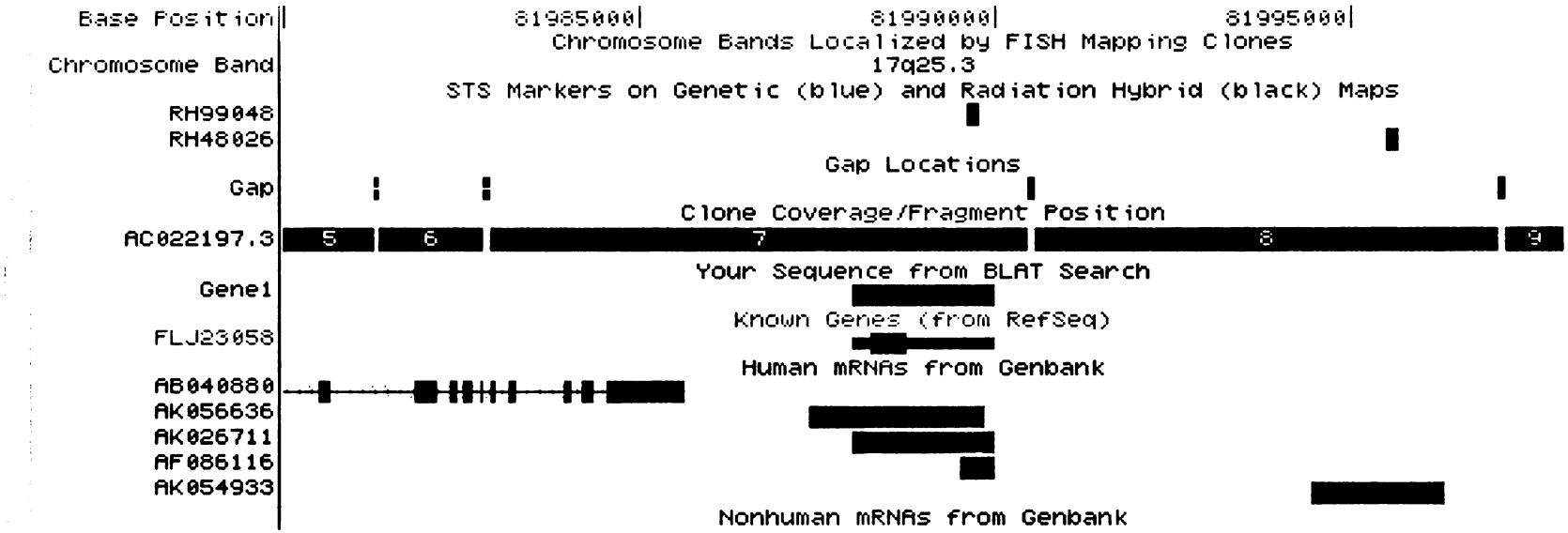

Figure 14. Chromosome location of gene 1. It is the FLJ23058, a novel gene represented by stSG52023 (RH99048), an ear-expressed STS. AC022197 is the BAC 31B8 in which this gene is. FLJ23058 has one exon encoding a hypothetical protein of 180 amino acids. I.M.A.G.E. 2168160 is part of gene 1.

Figure 15. The full-length cDNA and predicted amino acid sequences of gene 1. It represented by an ear-expressed STS, stSG52023. At 5' start site, there is no KOZAC conservative sequence (ACCATGG) (Kozak, 1986) suggesting that this is only part of the whole gene.

```
ttttggtttc ctttggaaat gcactgttct cagcccagct
gggttccaac gggggcacct gggacgacag aggcatctcg
gggcaggggg cagaggccac gggagggtca ggtgggaccc
ttgacggcac cctctgatct cttgggggga cgaccgtgta
agatgaaagt cggtcaagtt tatttgcttt cagtgcatct
cctagaaaag aagtgttgga gcagggggtg GTG

234 ATGaggggcccgtggggaaaggattcaagaggcaaagcccagacc
     M  R  G  P  W  G  K  D  S  R  G  K  A  Q  T
279 agggagtgtgacaacagccgtgagcatctcacctgtgttcaaaac
     R  E  C  D  N  S  R  E  H  L  T  C  V  Q  N
324 aagacaaagacgaacaaatattttaaagtattgataagaaaaagc
     K  T  K  T  N  K  Y  F  K  V  L  I  R  K  S
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     N  V  W  I  V  S  A  E  S  Y  S  N  L  Y  L
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     I  S  V  S  G  A  S  W  S  E  A  K  E  S  G
459 ctccacccagagcagggaggggctggcccctcgcccccctcaccc
     L  H  P  E  Q  G  G  A  G  P  S  P  P  S  P
504 tcgccgccctggcacactcgggaagcagggcccagctctgagccc
     S  P  P  W  H  T  R  E  A  G  P  S  S  E  P
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     G  A  Y  E  G  R  F  T  V  P  P  D  S  L  L
684 acccccaagcagaatgcatgcaaaagacacccctttcccaccca
     T  P  K  Q  N  A  C  K  R  H  P  F  S  H  P
729 ccttattggtgcccccaaacccctggcctgctgcgtagatggtgg
     P  Y  W  C  P  Q  T  P  G  L  L  R  R  W  W
774 tga 776 ggcc
     *
aggccagcag tgctgtggcc aggggagaag aaaataaaac
gcaggccctg cccttgggga aggcccctttt ctgggccccc
ttttccacca gccaaatctg cctggccctc ggacccctct
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tgccacttgg tggggcagat ggcctgatgg gctagtgctt
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atcctggccc caggtgcaag cagcacccct cgagggctct
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ggcccctccc ctctcaccgt ggttaacgtg acgaaggcac
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tttttcaatg taaacactaa aaacaaaatg gacaaaaaaa
cacacaaagg ttttatgaac agcagactct atgtaaaggc
attttagtat caaatttttt attgataact tgcttaagaa
taaatatatg gactattgta aaaaaaaaaa aaaaaaaaaa aa

with 2.9kb from stomach was ordered and sequenced. This cDNA sequence is homologous to the reported 'full length' mRNA, AF217993, which has one exon, encoding a 193 aa (Figure 16). The whole block of this cDNA is completely homologous to the genomic sequence. Primer pair (kfl135/1136) was designed to sequence the coding region on affected individual 406. No variation was found compared to the reference sequences from NCBI.

Unigene cluster Hs. 104767 represented by stSG46575 was reported by the NCBI. It has a full-length mRNA BC014642 (GeneBank accession number) of 2.775kb with an open reading frame encoding 780 aa. This aa sequence was used to search a conserved-domain database (http://www.ncbi.nlm.nih.gov/Structure/cdd/). This gene appears to have a transmembrane amino acid transporter domain. The BC014642 was used to blast the mouse EST database (http://www.ncbi.nlm.nih.gov/blast/), and the mouse homolog BI656563 was found. This belongs to the mouse unigene cluster Mm3310. This mouse unigene cluster is expressed in one out of the five mouse embryonic cochlear stages based on the Inner Ear Gene expression Database from the Corey lab (http://www.mgh.harvard.edu/depts/coreylab/genomics.html).

Primer pairs (kfl123/1124, kfl125/1126, kfl127/1128, kfl129/1130) were designed from the cDNA sequence. A reverse transcript from hybrid cell lines #15 was made. The sequences of the hybrid cDNAs showed no variation from the reference cDNA sequence of BC014642, therefore, this part of the transcript unit is not considered to be a possible candidate for DFNA20.

Unigene cluster Hs.287797 and Hs. 104767 are in the same transcribing orientation (Figure 16). It is likely that they belong to one gene with alternative splicing products. The primer pair kf1195/1197 was designed from AF217993 and BC014642, PCR on both hybrid cell line #15 and #8 gives about 1.4kb product, which bridged these two cDNA (Figure 17). This suggests that they belong to the same gene. The sequence of this whole cDNA showed no significant variation (Table 10).

Using the reverse primer of sts-T49250 as 5' end ^{32}P labeled probe, a multiple tissue northern blot showed that this gene probably has alternative splicing with three sizes (2.5, 5.5 and 6.3kb) of mRNA in heart, skeletal muscle and liver (Figure 18).

Gene 2, a novel gene of 4.305kb, encodes 1118 aa (Figure 19). It is part of the full transcript, which is 6.3 kb based on the multiple tissue northern blot. It means alternative splicing is indeed going on in this gene. To date, we have not found the full-length cDNA. EST information clearly indicates two poly A sites, also one splice variant with 4 exons spliced out (http://genome.ucsc.edu).

5. Characterization of gene 3-----Hs.232219:

This has been merged with gene 2 but should not have been since the polyA is on the opposite strand. There are only two I.M.A.G.E clones in this unigene cluster, one from the Morton fetal cochlear library, which is how this unigene cluster was identified, the

Table 10. Summary of sequence variations on the novel gene 2

Position (kb)	2.837	3.045
Reference	G	A
306 normal chromosome	C	G
306 mutant chromosome	G	A
406 (affected individual)	G/G	A/A
Amino acid change	Ala→ Gly (nonpolar)	no
Comments	N.V.	N.V.

Note: N.V. represents normal variant. Both Alanine and Glycine are nonpolar neutral amino acids. 406 is one of the affected individuals in MSUDF1 family (Figure 7), a son of the proband. It is expected that one copy of his chromosomes carries the disease gene.

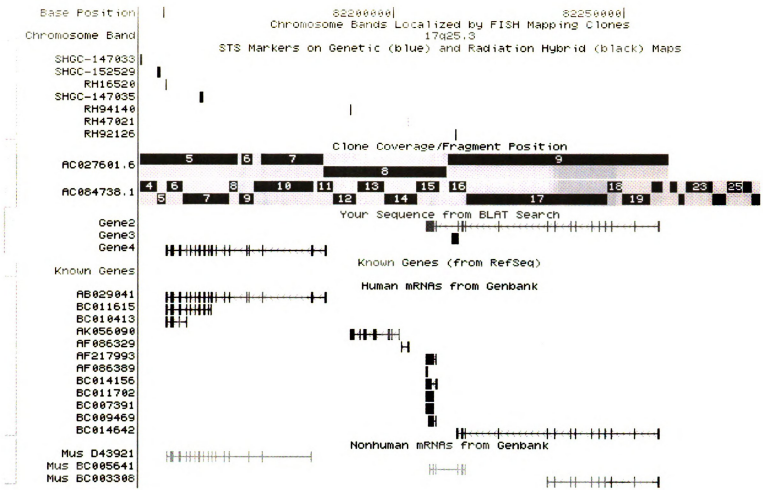

Figure 16. Chromosome location of the other three novel genes, gene 2, 3 and 4. Gene 2 brings unigene clusters: Hs. 287797 and Hs.104767 together to form one novel gene with 16 exons in total. Gene 3 is a novel gene represented by unigene Hs.232219, it has one predicted exon. Gene 4 is a novel gene derived from AB029041, it has 25 exons.

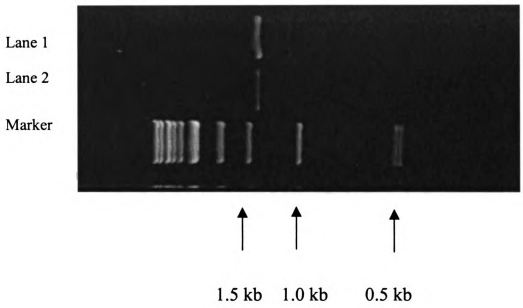

Figure 17. PCR product from the cDNA of both normal (Lane 2) and affected (lane 1) hybrids using the primers (kf1195/kf1197) on the BC014642 and AF217993.

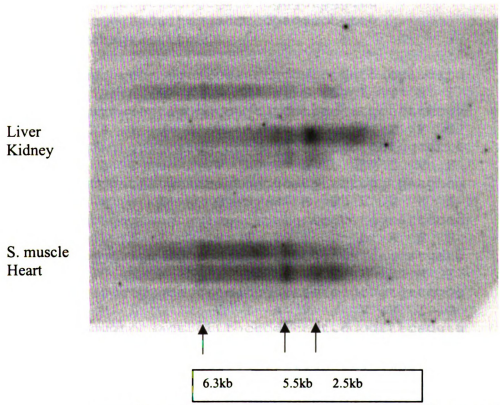

Figure 18. Northern blot hybridization using 5' end ^{32}P-labeled reverse primer (20-mer) of sts-T49250.

Figure 19. The full–length cDNA and predicted amino acid sequences of gene 2. It represented by sts-T49250 and stSG46575. It is 4.305kb long encoding 1118 amino acids. At 5' start site, a reasonable good KOZAC sequence (ACTATGA) compared to the complete conservative KOZAC sequence (ACCATGG) (Kozak, 1986) was found meaning this sequence might be a full transcript of a gene or part of a full transcript based on the northern blot.

```
tgtccctagctggctctgcggctcttccgggtctgggctc
ggagattacaggcggcccgcgaggccgagcgagggacgca
tggccctgaggcggccgcagggcttggcggggtccggagg
ttgacctcgcccccgcagccggccttcgaggctgcctcct
ccaggcagcctctggggcccgcgcccgcgcctgctcaggc
tcccgtgttcaggctgcccatcccctccccaccggcgtcc
cggacgttgggacctgtgaccgtggcctcgggctgggctt
ccaaagccggccgcagcccggcgacccccgaggcctctcg
ccccgggcccctagacctctcACT

346 ATGAccgcggccgccgcctccaactgggggctgatcacgaacatc
     M  T  A  A  A  A  S  N  W  G  L  I  T  N  I
391 gtgaacagcatcgtaggggtcagtgtcctcaccatgcccttctgc
     V  N  S  I  V  G  V  S  V  L  T  M  P  F  C
436 ttcaaacagtgcggcatcgtcctgggggcgctgctcttggtcttc
     F  K  Q  C  G  I  V  L  G  A  L  L  L  V  F
481 tgctcatggatgacgcaccagtcgtgcatgttcttggtgaagtcg
     C  S  W  M  T  H  Q  S  C  M  F  L  V  K  S
526 gccagcctgagcaagcggaggacctacgccggcctggcattccac
     A  S  L  S  K  R  R  T  Y  A  G  L  A  F  H
571 gcctacgggaaggcaggcaagatgctggtggagaccagcatgatc
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616 gggctgatgctgggcacctgcatcgccttctacgtcgtgatcggc
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     D  L  G  S  N  F  F  A  R  L  F  G  F  Q  V
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     G  G  T  F  R  M  F  L  L  F  A  V  S  L  C
751 atcgtgctcccgctcagcctgcagcggaacatgatggcctccatc
     I  V  L  P  L  S  L  Q  R  N  M  M  A  S  I
796 cagtccttcagcgccatggccctcctcttctacaccgtgttcatg
     Q  S  F  S  A  M  A  L  L  F  Y  T  V  F  M
841 ttcgtgatcgtgctctcctctctcaagcacggcctcttcagtggg
     F  V  I  V  L  S  S  L  K  H  G  L  F  S  G
886 cagtggctgcggcgggtcagctacgtccgctgggagggcgtcttc
     Q  W  L  R  R  V  S  Y  V  R  W  E  G  V  F
931 cgctgcatccccatcttcggcatgtccttcgcctgccagtcccag
     R  C  I  P  I  F  G  M  S  F  A  C  Q  S  Q
976 gtgctgcccacctacgacagcctggatgagccgtcagtgaaaacc
     V  L  P  T  Y  D  S  L  D  E  P  S  V  K  T
```

```
1021 atgagctccatatttgcttcctcccttaatgtggtcaccaccttc
      M  S  S  I  F  A  S  S  L  N  V  V  T  T  F
1066 tacgtcatggtggggttttttcggctacgtcagcttcaccgaggcc
      Y  V  M  V  G  F  F  G  Y  V  S  F  T  E  A
1111 acggccggcaacgtgctcatgcactttccctccaacctggtgacg
      T  A  G  N  V  L  M  H  F  P  S  N  L  V  T
1156 gagatgctccgtgtgggcttcatgatgtcagtggctgtgggcttc
      E  M  L  R  V  G  F  M  M  S  V  A  V  G  F
1201 cccatgatgatcctgccatgcaggcaggccctgagcacgctgctg
      P  M  M  I  L  P  C  R  Q  A  L  S  T  L  L
1246 tgtgagcagcagcaaaaagatggcacctttgcagcaggggctac
      C  E  Q  Q  Q  K  D  G  T  F  A  A  G  G  Y
1291 atgccccctctccggtttaaagcacttaccctctctgtggtgttt
      M  P  P  L  R  F  K  A  L  T  L  S  V  V  F
1336 ggaaccatggttggtggcatccttatccccaacgtggagaccatc
      G  T  M  V  G  G  I  L  I  P  N  V  E  T  I
1381 ctgggcctcacaggagcgaccatgggaagcctcatctgcttcatc
      L  G  L  T  G  A  T  M  G  S  L  I  C  F  I
1426 tgcccggcgctgatctacaagaaaatccacaagaacgcactttcc
      C  P  A  L  I  Y  K  K  I  H  K  N  A  L  S
1471 tcccaggtggtgctgtgggtcggcctgggcgtcctggtggtgagc
      S  Q  V  V  L  W  V  G  L  G  V  L  V  V  S
1516 actgtcaccacactgtctgtgagcgaggaggtccccgaggacttg
      T  V  T  T  L  S  V  S  E  E  V  P  E  D  L
1561 gcagaggaagcccctggcggccggcttggagaggccgagggtttg
      A  E  E  A  P  G  G  R  L  G  E  A  E  G  L
1606 atgaaggtggaggcagcgcggctctcagcccaggatccggttgtg
      M  K  V  E  A  A  R  L  S  A  Q  D  P  V  V
1651 gccgtggctgaggatggccgggagaagccgaagctgccgaaggag
      A  V  A  E  D  G  R  E  K  P  K  L  P  K  E
1696 agagaggagctggagcaggcccagatcaaggggcccgtggatgtg
      R  E  E  L  E  Q  A  Q  I  K  G  P  V  D  V
1741 cctggacgggaagatggcaaggaggcaccggaggaggcacagctc
      P  G  R  E  D  G  K  E  A  P  E  E  A  Q  L
1786 gatcgccctgggcaagggattgctgtgcctgtgggcgaggcccac
      D  R  P  G  Q  G  I  A  V  P  V  G  E  A  H
1831 cgccacgagcctcctgttcctcacgacaaggtggtggtagatgaa
      R  H  E  P  P  V  P  H  D  K  V  V  V  D  E
1876 ggccaagaccgagaggtgccagaagagaacaaacctccatccaga
      G  Q  D  R  E  V  P  E  E  N  K  P  P  S  R
1921 cacgcgggcggaaaggctccaggggtccagggccagatggcgccg
      H  A  G  G  K  A  P  G  V  Q  G  Q  M  A  P
1966 cctctgcccgactcagaaagagagaaacaagagccggagcaggga
      P  L  P  D  S  E  R  E  K  Q  E  P  E  Q  G
2011 gaggttgggaagaggcctggacaggcccaggccttggaggaggcg
      E  V  G  K  R  P  G  Q  A  Q  A  L  E  E  A
```

```
2056 ggtgatcttcctgaagatccccagaaagttccagaagcagatggt
      G  D  L  P  E  D  P  Q  K  V  P  E  A  D  G
2101 cagccagctgtccagcctgcaaaggaggacctggggccaggagac
      Q  P  A  V  Q  P  A  K  E  D  L  G  P  G  D
2146 aggggcctgcatcctcggccccaggcagtgctgtctgagcagcag
      R  G  L  H  P  R  P  Q  A  V  L  S  E  Q  Q
2191 aacggcctggcggtgggtggaggggaaaaggccaaggggggaccg
      N  G  L  A  V  G  G  G  E  K  A  K  G  G  P
2236 ccgccaggcaacgccgccggggacacagggcagcccgcagaggac
      P  P  G  N  A  A  G  D  T  G  Q  P  A  E  D
2281 agcgaccacggtgggaagcctcccctcccagcggagaagccggct
      S  D  H  G  G  K  P  P  L  P  A  E  K  P  A
2326 ccagggcctgggctgccgcccgagcctcgcgagcagagggacgtg
      P  G  P  G  L  P  P  E  P  R  E  Q  R  D  V
2371 gagcgagcgggtggaaaccaggcggccagccagctggaggaagct
      E  R  A  G  G  N  Q  A  A  S  Q  L  E  E  A
2416 ggcagggcggagatgctggaccacgccgtcctgcttcaggtgatc
      G  R  A  E  M  L  D  H  A  V  L  L  Q  V  I
2461 aaagaacagcaggtgcagcaaaagcgcttgctggaccagcaggag
      K  E  Q  Q  V  Q  Q  K  R  L  L  D  Q  Q  E
2506 aagctgctggcggtgatcgaggagcagcacaaggagatcccccag
      K  L  L  A  V  I  E  E  Q  H  K  E  I  P  Q
2551 cagaggcaggaggacgaggaggataaacccaggcaggtggaggtg
      Q  R  Q  E  D  E  E  D  K  P  R  Q  V  E  V
2596 catcaagagcccggggcagcggtgcccagaggccaggaggcccct
      H  Q  E  P  G  A  A  V  P  R  G  Q  E  A  P
2641 gaaggcaaggccagggagacggtggagaatctgcctcccctgcct
      E  G  K  A  R  E  T  V  E  N  L  P  P  L  P
2686 ttggaccctgtcctcagagctcctgggggccgccctgctccatcc
      L  D  P  V  L  R  A  P  G  G  R  P  A  P  S
2731 caggaccttaaccagcgctccctggagcactctgaggggcctgtg
      Q  D  L  N  Q  R  S  L  E  H  S  E  G  P  V
2776 ggcagagaccctgctggccctcctgacggcggccctgacacagag
      G  R  D  P  A  G  P  P  D  G  G  P  D  T  E
2821 cctcgggcagcccagggcaagctgagagatggccagaaggatgcc
      P  R  A  A  Q  G  K  L  R  D  G  Q  K  D  A
2866 gcccccagggcagctggcactgtgaaggagctccccaagggcccg
      A  P  R  A  A  G  T  V  K  E  L  P  K  G  P
2911 gagcaggtgcccgtgccagaccccgccagggaagccgggggccca
      E  Q  V  P  V  P  D  P  A  R  E  A  G  G  P
2956 gaggagcgcctcgcagaggaattccctgggcaaagtcaggacgtt
      E  E  R  L  A  E  E  F  P  G  Q  S  Q  D  V
3001 actggcggttcccaagacaggaaaaaacctgggaaggaggtggca
      T  G  G  S  Q  D  R  K  K  P  G  K  E  V  A
3046 gccactggcaccagcattctgaaggaagccaactggctcgtggca
      A  T  G  T  S  I  L  K  E  A  N  W  L  V  A
```

```
3091 gggccaggagcagagacgggggaccctcgcatgaagcccaagcaa
      G  P  G  A  E  T  G  D  P  R  M  K  P  K  Q
3136 gtgagccgagacctgggccttgcagcggacctgcccggtggggcg
      V  S  R  D  L  G  L  A  A  D  L  P  G  G  A
3181 gaaggagcagctgcacagccccaggctgtgttacgccagccggaa
      E  G  A  A  A  Q  P  Q  A  V  L  R  Q  P  E
3226 ctgcgggtcatctctgatggcgagcagggtggacagcagggccac
      L  R  V  I  S  D  G  E  Q  G  G  Q  Q  G  H
3271 cggctggaccatggcggtcacctggagatgagaaaggcccgcggg
      R  L  D  H  G  G  H  L  E  M  R  K  A  R  G
3316 ggggaccatgtgcctgtgtcccacgagcagccgagaggcggggag
      G  D  H  V  P  V  S  H  E  Q  P  R  G  G  E
3361 gacgctgctgtccaggagcccaggcagaggccagagccagagctg
      D  A  A  V  Q  E  P  R  Q  R  P  E  P  E  L
3406 gggctcaaacgagctgtcccgggggggccagaggccggacaatgcc
      G  L  K  R  A  V  P  G  G  Q  R  P  D  N  A
3451 aagcccaaccgggacctgaaactgcaggctggctccgacctccgg
      K  P  N  R  D  L  K  L  Q  A  G  S  D  L  R
3496 aggcgacggcgggaccttggccctcatgcagagggtcagctggcc
      R  R  R  R  D  L  G  P  H  A  E  G  Q  L  A
3541 ccgagggatggggtcattggccttaacccccctgcctgatgtccag
      P  R  D  G  V  I  G  L  N  P  L  P  D  V  Q
3586 gtgaacgacctccgtggcgccctggatgcccagctccgccaggct
      V  N  D  L  R  G  A  L  D  A  Q  L  R  Q  A
3631 gcgggggggagctctgcaggtggtccacagccggcagcttagacag
      A  G  G  A  L  Q  V  V  H  S  R  Q  L  R  Q
3676 gcgcctgggcctccagaggagtcctag 3702
      A  P  G  P  P  E  E  S  *

cacctgctggccatgagggccacgccagccactgccctcc
tcggccagcagcaggtctgtctcagccgcatcccagccaa
actctggaggtcacactcgcctctccccagggtttcatgt
ctgaggccctcaccaagtgtgagtgacagtataaaagatt
cactgtggcatcgtttccagaatgttcttgctgtcgttct
gttgcagctcttagtctgaggtcctctgacctctagactc
tgagctcactccagcctgtgaggagaaacggcctccgctg
cgagctggctggtgcactcccaggctcaggctggggagct
gctgcgtctgtggtcaggcctcctgctcctgccagggagc
acgcgtggtcttcgggttgagctcgcccgtgcgtggaggt
gcgcatggctgctcatggtcccaacacaggctactgtgag
agccagcatccaaccccacgcttgcagtgactcagaatga
taattattatgactgtttatcgatgcttcccccagtgtgg
tagaaagtcttgaataaacacttttgccttcacccaaaaa
aaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaa
```

other from human brain, which has 1.5 kb insert. The I. M. A. G. E 34455 from brain was obtained from Research Genetics and sequenced. The full sequence of the insert is 1.5kb with poly (A) tail. Its genomic location is shown on Figure 16. Its transcription direction is the opposite of gene 2.

Using the insert of the IMAGE 2483412 from the Morton fetal cochlear library as a probe, a multiple tissue northern blot showed that this gene is highly expressed in skeletal muscle and is approximately 1.75kb long (Figure 20).

5'RACE was used to find the 5' end sequence, which proved that the whole sequence is about 1.7kb in length. It was fully homologous with the genomic sequence with an ORF of 459 nucleotides encoding 152 aa (Figure 21).

Sequencing data on the affected conversion cell cDNA showed no nucleotide changes at all. There was no homology to any known gene.

6. Characterization of gene 4-----Hs. 209646 represented by stSG9053

In 1999, Kikuno's research group in Japan (Kikuno, et al. 1999) reported the coding sequences of 100 new cDNA clones including 27 clones from the fetal brain, which expressed large proteins in vitro. KIAA1118 is one of the reported genes, located on 17q25 on the sequenced BACs, 455O6 and 11J14 (Figure 16). It belongs to the unigene

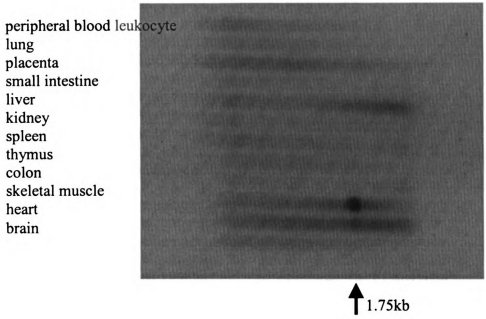

Figure 20. Northern blot hybridization using random primer-labeled cDNA fragment (280bp long) from the Morton fetal cochlear I.M.A.G.E cDNA clone 2483412.

Figure 21. The full-length cDNA and predicted amino acid sequences of gene 3. It is 1.75kb encoding 152 aa. It is from unigene cluster Hs.232219 and has a reasonable KOZAC sequene (AACATGG) at the 5' start site compared to the complete conservative sequence (ACCATGG) (Kozak, 1986).

```
ctgctcgtcccgttgagaggcatacggaagaaaggcagaa
Taggaaaccgtgaaaagtcttttcagaaagaagaaaatc
Aaaagaatcaaacaggttcaggtaacagagcataggaatt
ggaaccaacaAAC

134 ATGGccagactaaaaccggagacaaaaccccacatgcccgaactc
     M  A  R  L  K  P  E  T  K  P  H  M  P  E  L
179 ctgaagtggctcagagctccgggagcccaggggcgccttttggc
     L  K  W  L  R  A  P  G  A  Q  G  A  P  F  G
224 tctgccttccgcgctgcaggtgcaaggagggcgagctgcacctgg
     S  A  F  R  A  A  G  A  R  R  A  S  C  T  W
269 agaagccaggccgcagcgactgggacgtggctgctgcacctgccc
     R  S  Q  A  A  A  T  G  T  W  L  L  H  L  P
314 tccctcggaccggctgtcctgaggccacagggcaagatgcgcggg
     S  L  G  P  A  V  L  R  P  Q  G  K  M  R  G
359 caggaagcgtgtgtcttacgaacatcctgggaggtgcccgtgcca
     Q  E  A  C  V  L  R  T  S  W  E  V  P  V  P
404 gggctggcccggagctcccacacctgctcccaggggcttggcaca
     G  L  A  R  S  S  H  T  C  S  Q  G  L  G  T
449 ggaggctggagcggctgtgccctgcgacccccacacagatccctg
     G  G  W  S  G  C  A  L  R  P  P  H  R  S  L
494 gaacagaccctctcaatgctgaggcacacgtggaccccgcatggg
     E  Q  T  L  S  M  L  R  H  T  W  T  P  H  G
539 gcagggttgaccatcttgagagaaggcttggacactgccaccaaa
     A  G  L  T  I  L  R  E  G  L  D  T  A  T  K
584 tgcacgtga 592
     C  T  *
cttttggtgagcgggggcccagccacacaccctcagaggg
cgggtggacccctagcctgggagaggacatggcacagaag
cctgtccccagcaggctcacagacggggctgctgctaccc
aactgcatgaggccacaaggaggggtcgcaaaaagccaac
agctgagacccaaggggcgagaagtttggaggaggattca
gagcaagttttattctcaggagagcaaaggcgtaaggagc
tttcctggagggacaggtgacagatccctgtagatgaaca
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tgggccagggctatgctaggaccagggcgggcacagccac
ccccaaactgggtccccttggggcacagccttaaaataaa
cctccacatctgggtgcaggcacgcccacacctggctggc
tttaaatactccgagtgttaaaactgttaacaaacctgct
ttaaagacgaaatggtaatcacaagccaagtgacaatgcc
gcaggtgttttaaaggggagcaagggagataaaacggtaa
tcacaagccatgtgacgatgccgcaggtgtttaaagggga
```

gtaaggcagataaaaggttcccagaaagcaagctgctgat
gggaagggagcagaaagctctccgaggggtctgctcagca
ggcggctggaaagagaaggggggcctggcagggctcccga
ggatgacctgggcatcaccccagggctgggggaggggggct
gccgctccctgggcctgaggcaggcggctgtagggcggag
gccttaccctccagctggctggccgcctggtttccacccg
ctcgctccacgtccctctgctcgcgaggctcgggcggcag
cccaggccctggagccggcttctccgctgggaggggaggc
ttcccacctgcacacacggtgaagactcagaaggttttgc
aggaaagtcaagggagggtttgggggttgggggacagaa
ggcaagggcgggacaaaaggaagtggcagatgtttctaaa
gtgacacccccgtcagcacagtttatattcaacctgatga
ttactagcgaagcaataaaaaaatatttaacaacctcaaa
aaaaaaaaaaaaaaaaaaaaaaaaaaaaa

cluster Hs.209646 represented by stSG9053. It has a wide tissue expression including brain, colon, breast, eye, nervous system, lung, placenta, and skin. Even though the stSG9053 was not found in the human fetal cochlear library based on PCR, KIAA1118 gene contains important conserved domains including a myosin tail, ERM and ATPase binding site, which are proved to be important in ear function (Redowicz, 1999). These features make this gene a strong candidate gene for DFNA20. The mouse homolog, D43921, in the mouse unigene cluster Mm45540, is 3.566kb long with 25 exons. The Inner Ear Gene Expression Database from the Corey Lab in Harvard Medical School shows this mouse unigene to be expressed in three of the five mouse embryonic stages.

Based on the ESTs on the UCSC database and the mouse homolog D43921, 25 exons are predicted from this KIAA1118 gene (Figure 22).

Sequencing data both on genomic and cDNA shows no significant variation (Table 11).

Figure 22. The full-length cDNA and predicted aa sequences of KIAA1118. It is 3.673kb encodeing 1083 aa. It has conserved KOZAC sequence (ACCATGG) (Kozak, 1986) at the 5' start site.

```
gacgggctgg ggaggtggtg ccaggctggt tgctagctgc
cgccgctgct caccccggcc gtccggccgc ttcattgtca
ccgggcctcg gggcagtgtt gggggtggcg gggcgccagg
tcgccgaccc gaggtttcgg cgcgactggt tccgcatccc
ctccccgccg gccgaatctg caggccccgc gcgccaggca
ggcttcgcgg ctgcgccccc ggcccgcgca ggacctgcct
tgtCCACC

249 ATGaaaggcacccgggccatcggcagcgtcccggagcgcagccca
     M  K  G  T  R  A  I  G  S  V  P  E  R  S  P
294 gcaggtgtggacctgagtctgacaggtctccctccgcctgtgtcc
     A  G  V  D  L  S  L  T  G  L  P  P  P  V  S
339 cggcgtcctggcagtgccgccaccaccaagcccatcgtccgctct
     R  R  P  G  S  A  A  T  T  K  P  I  V  R  S
384 gtctccgtggtcacaggcagcgagcagaagaggaaggtgctggag
     V  S  V  V  T  G  S  E  Q  K  R  K  V  L  E
429 gccacagggcctgggggctcccaggccatcaacaaccttagaaga
     A  T  G  P  G  G  S  Q  A  I  N  N  L  R  R
474 tccaacagcaccacgcaggtcagccagcctcggagcggctccccc
     S  N  S  T  T  Q  V  S  Q  P  R  S  G  S  P
519 aggccaacggagcccacagacttcctgatgctcttcgagggcagc
     R  P  T  E  P  T  D  F  L  M  L  F  E  G  S
564 cccagtgggaaaaagaggcctgccagcctgagcacagcccccagc
     P  S  G  K  K  R  P  A  S  L  S  T  A  P  S
609 gagaagggagccacctggaacgtcctggatgaccagccccggggc
     E  K  G  A  T  W  N  V  L  D  D  Q  P  R  G
654 ttcaccttgccatccaatgcccggagttccagtgcccttgactca
     F  T  L  P  S  N  A  R  S  S  S  A  L  D  S
699 ccagcgggcccgcggaggaaagaatgcaccgtggccctggccccc
     P  A  G  P  R  R  K  E  C  T  V  A  L  A  P
744 aacttcactgctaacaacaggagcaacaagggagcagtgggcaac
     N  F  T  A  N  N  R  S  N  K  G  A  V  G  N
789 tgcgtcaccaccatggtgcacaaccgctacacccccctcggagagg
     C  V  T  T  M  V  H  N  R  Y  T  P  S  E  R
834 gcgcctccgctcaagagctccaaccagactgccccctccctcaac
     A  P  P  L  K  S  S  N  Q  T  A  P  S  L  N
879 aacatcatcaaggcagccacctgtgagggcagtgagagcagcggc
     N  I  I  K  A  A  T  C  E  G  S  E  S  S  G
924 tttgggaagctgccgaagaatgtctccagtgccacccactcagcc
     F  G  K  L  P  K  N  V  S  S  A  T  H  S  A
969 cggaacaatactgggggcagcacgggcttgcccaggcggaaggag
      R  N  N  T  G  G  S  T  G  L  P  R  R  K  E
```

```
1014 gtgacggaggaggaggctgagaggtttatccaccaggtgaaccag
      V  T  E  E  E  A  E  R  F  I  H  Q  V  N  Q
1059 gccgctgtcaccatccagcgctggtaccggcaccaggtgcagcgg
      A  A  V  T  I  Q  R  W  Y  R  H  Q  V  Q  R
1104 cgcggagcaggagctgcccgcctggagcacttgcttcaggccaag
      R  G  A  G  A  A  R  L  E  H  L  L  Q  A  K
1149 cgggaggagcagcggcagcggtcaggcgaggggaccctcttggac
      R  E  E  Q  R  Q  R  S  G  E  G  T  L  L  D
1194 ctgcaccagcagaaagaggcagccaggaggaaggcccgggaggag
      L  H  Q  Q  K  E  A  A  R  R  K  A  R  E  E
1239 aaggcacgccaagccaggcgagcagccattcaggagctgcaacag
      K  A  R  Q  A  R  R  A  A  I  Q  E  L  Q  Q
1284 aaacgagccctgagagcccagaaggcgagcactgccgagcgtggg
      K  R  A  L  R  A  Q  K  A  S  T  A  E  R  G
1329 ccacctgagaatcccagggagaccagagtgccaggaatgcggcag
      P  P  E  N  P  R  E  T  R  V  P  G  M  R  Q
1374 cctgctcaggagctgtcccccacgccaggcggcactgcccaccag
      P  A  Q  E  L  S  P  T  P  G  G  T  A  H  Q
1419 gccctcaaggccaacaatgctggtggtggcctccctgctgcaggc
      A  L  K  A  N  N  A  G  G  G  L  P  A  A  G
1464 cccggagaccgctgcctgcccacctccgactcatccccagaacca
      P  G  D  R  C  L  P  T  S  D  S  S  P  E  P
1509 cagcagcctccagaggacaggacgcaggacgttcttgcccaggat
      Q  Q  P  P  E  D  R  T  Q  D  V  L  A  Q  D
1554 gcagctggggacaacctggagatgatggccccgagcaggggagc
      A  A  G  D  N  L  E  M  M  A  P  S  R  G  S
1599 gccaagtccaggggccactggaggagctgctgcacacactgcag
      A  K  S  R  G  P  L  E  E  L  L  H  T  L  Q
1644 ctgctggagaaggagccggacgcgctgccccgccccaggacccat
      L  L  E  K  E  P  D  A  L  P  R  P  R  T  H
1689 cacaggggcagatacgcctgggccagcgaggtgaccacggaggat
      H  R  G  R  Y  A  W  A  S  E  V  T  T  E  D
1734 gacgccagctctctgacagctgacaacttggagaaatttggaaaa
      D  A  S  S  L  T  A  D  N  L  E  K  F  G  K
1779 ctcagtgcgttccccgaacctcctgaggatgggacgctgctatcg
      L  S  A  F  P  E  P  P  E  D  G  T  L  L  S
1824 gaggccaagctacaaagcatcatgagcttcttggacgagatggag
      E  A  K  L  Q  S  I  M  S  F  L  D  E  M  E
1869 aagtctgggcaggaccagctggactcccagcaggaggggtgggtg
      K  S  G  Q  D  Q  L  D  S  Q  Q  E  G  W  V
1914 ccggaggcggggccggggcccctggagctggggtccgaggtgagc
      P  E  A  G  P  G  P  L  E  L  G  S  E  V  S
1959 acgtctgtgatgcggctgaagctggaggtggaggagaagaagcag
      T  S  V  M  R  L  K  L  E  V  E  E  K  K  Q
2004 gccatgctgctgctgcagagagcgctggcgcagcagcgagacctc
      A  M  L  L  L  Q  R  A  L  A  Q  Q  R  D  L
```

```
2049 acggcccggcgggtcaaggagacagagaaggcactgagccggcag
      T  A  R  R  V  K  E  T  E  K  A  L  S  R  Q
2094 ctgcagcggcagagggagcactacgaggccaccatccagcggcac
      L  Q  R  Q  R  E  H  Y  E  A  T  I  Q  R  H
2139 ttggccttcattgaccagctgattgaggacaagaaggtcctgagt
      L  A  F  I  D  Q  L  I  E  D  K  K  V  L  S
2184 gaaaagtgcgaggctgtggtggccgagctgaagcaggaggaccag
      E  K  C  E  A  V  V  A  E  L  K  Q  E  D  Q
2229 agatgcaccgagcgtgtggcccaggcacaggcgcagcacgagctg
      R  C  T  E  R  V  A  Q  A  Q  A  Q  H  E  L
2274 gagattaaaaaactcaaagaattaatgagcgccaccgagaaagcc
      E  I  K  K  L  K  E  L  M  S  A  T  E  K  A
2319 cgccgggagaagtggatcagtgagaaaaccaagaagatcaaggag
      R  R  E  K  W  I  S  E  K  T  K  K  I  K  E
2364 gtcactgtccgaggtctggagcccgagatccagaagctgattgca
      V  T  V  R  G  L  E  P  E  I  Q  K  L  I  A
2409 aggcacaagcaggaagtgcggaggctcaagagcctgcacgaggcg
      R  H  K  Q  E  V  R  R  L  K  S  L  H  E  A
2454 gagctgctgcagtcggatgagcgggcctcgcagcgctgcctgcgc
      E  L  L  Q  S  D  E  R  A  S  Q  R  C  L  R
2499 caggccgaggagctgcgggagcagctggagcgggagaaggaggcg
      Q  A  E  E  L  R  E  Q  L  E  R  E  K  E  A
2544 ctgggccagcaggagcgcgaacgtgctcggcagcggttccagcag
      L  G  Q  Q  E  R  E  R  A  R  Q  R  F  Q  Q
2589 cacctggagcaggagcagtgggcgctgcagcagcaacggcagcgg
      H  L  E  Q  E  Q  W  A  L  Q  Q  Q  R  Q  R
2634 ctgtacagtgaggtggctgaggagagggagcggctgggccagcag
      L  Y  S  E  V  A  E  E  R  E  R  L  G  Q  Q
2679 gcagccaggcagcgggcggagctggaagagctgaggcagcagctg
      A  A  R  Q  R  A  E  L  E  E  L  R  Q  Q  L
2724 gaggagagcagctctgcactgacccgagccctgagggctgagttt
      E  E  S  S  S  A  L  T  R  A  L  R  A  E  F
2769 gagaagggcagggaggagcaggagcgccggcaccagatggagctg
      E  K  G  R  E  E  Q  E  R  R  H  Q  M  E  L
2814 aataccctgaagcagcagctggagctggagaggcaggcgtgggag
      N  T  L  K  Q  Q  L  E  L  E  R  Q  A  W  E
2859 gccggccgcaccaggaaggaggaggcgtggctgctgaaccgggaa
      A  G  R  T  R  K  E  E  A  W  L  L  N  R  E
2904 caggagctgagggaagaaatccggaaaggccgggacaaggagatt
      Q  E  L  R  E  E  I  R  K  G  R  D  K  E  I
2949 gagctggtcattcaccggctggaggccgacatggcgctggccaag
      E  L  V  I  H  R  L  E  A  D  M  A  L  A  K
2994 gaggagagtgagaaggctgccgagagccgcatcaagcgcttacgg
      E  E  S  E  K  A  A  E  S  R  I  K  R  L  R
3039 gacaagtacgaggccgagctctccgagctggagcagtcggagcgg
      D  K  Y  E  A  E  L  S  E  L  E  Q  S  E  R
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```
3084 aagcttcaggagcggtgctcggagctgaagggccagcttggggag
      K  L  Q  E  R  C  S  E  L  K  G  Q  L  G  E
3129 gccgagggcgagaatctgcgtctgcagggccttgtgcggcagaag
      A  E  G  E  N  L  R  L  Q  G  L  V  R  Q  K
3174 gagcgggcgctggaggatgcgcaggcggtgaacgagcagctttct
      E  R  A  L  E  D  A  Q  A  V  N  E  Q  L  S
3219 agcgagcgcagcaacctggcccaggtgatccgccaggagttcgag
      S  E  R  S  N  L  A  Q  V  I  R  Q  E  F  E
3264 gaccggctggcagcctctgaggaggagacgcggcaggccaaggcc
      D  R  L  A  A  S  E  E  E  T  R  Q  A  K  A
3309 gagctggccacgctgcaggcccgccagcagctggagctggaggag
      E  L  A  T  L  Q  A  R  Q  Q  L  E  L  E  E
3354 gtgcaccggagggtgaagacagccctcgcgaggaaggaggaggcc
      V  H  R  R  V  K  T  A  L  A  R  K  E  E  A
3399 gtgagcagcctccggacacaacatgaggctgcggtgaagcgggcc
      V  S  S  L  R  T  Q  H  E  A  A  V  K  R  A
3444 gaccacctggaggagctgctggagcagcacaggaggcccacgcca
      D  H  L  E  E  L  L  E  Q  H  R  R  P  T  P
3489 agtaccaagtga 3500
      S  T  K  *

ccagggatgc cgggaacact gtcgaagaac ggaaggcaga
ggacagaggc tggacgtggg cccagaggcc cacagggacg
cccacctgcc ccccacagag gctggtggtt gagatgccca
cggctaagca cctgtggctg cattttaaca gtaaaggagg
ccgttgtttt cagaaaaaaa aaaaaaaaaa aa
```

Table 11. Summary of the nucleotide variation in KIAA1118 gene based on both genomic and cDNA sequencing data.

Position	183	470	2079	2081	2267	2696
Reference sequence	G	A	G	A	C	G
306 normal chromosome	C	A	A	G	T	A
306 mutant chromosome	G	G	G	G	C	G
406 (affected individual)	G/G	G/G	G/G	G/G	C/C	----
Amino acid change	----	no	Ala--Thr	no	no	no
comments	N.V.	N.V.	N.V.	N.V.	N.V.	N.V.

Note: N.V. represents normal variant. Alanine is a nonpolar amino acid, threonine is uncharged polar amino acid. 406 is one of the affected individuals in MSUDF1 family. It is expected one of his two chromosomes carries the disease gene.

Discussion

Two methods were used to determine whether a novel gene is ear expressed: PCR on total human fetal cochlear phage cDNA and searching the Mouse Inner Ear Expression database from Corey lab in Harvard Medical School.

The human fetal cochlear cDNA library was constructed by the Research Group in NIDCD of NIH. The relative paucity of cDNAs greater than 1kb may reflect postmortem autolysis of cochlear RNAs obtained from human fetuses and the technical difficulty of performing dissections of the membranous labyrinths from the temporal bones. Nevertheless, it is a valuable reagent to look for genes that are preferentially expressed in the cochlea. However, because the library does not provide the full-length cDNA, some genes that are expressed in the human cochlea may be missed because probes near the 5' end of genes will not be expected to be reversed transcribed during the construction of the cDNA library using poly (T) primer.

For those novel genes that are not found in the cochlear cDNA library, the Inner Ear Gene Expression Database from the Corey Lab in the Harvard Medical School should help to determine whether the mouse homolog of the human novel gene possibly has cochlear expression in humans. This database provides a profile of mouse cochlear gene expression in five different embryonic stages using oligonucleotide arrays (Genechip, from Affimetrix) (http://www.mgh.harvard.edu/depts/coreylab/genomics.html).

If no mouse homolog is found for a particular human novel gene found, then a human transcript is less likely to be of interest.

For interesting transcripts, we obtained the full-length cDNA sequences either from the UCSC database (http://www.ucse.edu) or from the sequence of I.M.A.G.E cDNA clones (http://image.llnl.gov/). The I.M.A.G.E Consortium maintains the largest public cDNA clone collection in the world. These clones provide a major resource for researchers in the areas of gene discovery and gene expression. It is vital that the clones accurately represent the sequence data published for those clones in the NCBI GenBank database. This database was continually used in this project.

When any potential candidate transcript was identified, the public databases were searched and the longest cDNA were obtained. The sequences were then determined from the affected individual in the MSUDF1 family.

Twelve lane multiple northern blot profiles were used to determine the tissue expression and size. However, the commercially made blots did not give results that were consistent with the published data. Some genes that were supposed to be expressed in a certain tissues could not be detected. For example one I.M.A.G.E. clone in Hs.232219 unigene cluster is perhaps reported to be from brain, but the northern blot was negative in the brain lane. Possibly the mRNA from that tissue was degraded. Alternatively this gene has a very low expression level in brain.

5' RACE is one of the important ways to capture unknown sequences at 5' end (Cowell, et al. 1997). However, 5' RACE sensitivity is affected by the RT used and secondary structure at the 5'-end of the cDNA that may inhibit cDNA tailing. Double-stranded and hairpin structures impair tailing of cDNA by decreasing the availability of the 3'-OH for tailing. 5'RACE was used in this project to determine the 5' end sequence of gene 3, Hs.232219. This gave shorter sequences than that had been identified from amplification of the cDNA. This suggests that there might be a secondary structure that prevents the first strand cDNA synthesis, resulting in a shorter 5' unknown sequence.

Gene 1 seems to be part of a gene with alternative splicing or a previously unrecognized psuedogene, because there is a size difference between the northern blot and the length of the cDNA we have both from sequencing I. M. A. G. E clones and compiling cDNAs at UCSC database, and because there is no good convincing ORF and conserved KOZAC sequences.

The longest cDNA sequence information of Gene 2 is 4.305kb, but the northern blot gives the largest band of 6.3kb in length in skeletal muscle and heart. Most likely there are alternative splicing processes in this gene. One of the two sequence variants does not cause amino acid change, the other one changes the code of from Alanine to Glycine, but both are nonpolar neutral amino acids, and what is more important the mutant chromosome has the nucleotide G that is the same as the reference nucleotide G. With the data currently available, the full-length of this interesting transcript was not obtained if

the sizes from the multiple tissues northern blot are real. Therefore more work needs to be done to decide whether it is the DFNA20 gene in the MSUDF1 family,

With regard to gene 3, it is definitely an ear-expressed transcript. The sizes in the northern blot and that from I.M.A.G.E. and 5'RACE are about the same, the transcript gives a reasonably good ORF, and a good KOZAC sequence, the optimal sequence for initiation by eukaryotic ribosome. It is likely a real gene because there is a polyadenylation site, coincidence of size on northern blot and the full-length cDNA, and the conserved KOZAC sequence. However it is not the DFNA20 gene because there is no significant mutation in the affected family individual.

The sequence variants of KIAA1118 from MSUDF1 family mostly do not cause amino acid changes, except at position of 2079 where a nucleotide g to a change causes an amino acid Alanine (nonpolar neutral aa) to Threonine (uncharged polar aa) change, but on the normal chromosome. The mutant hybrid of proband 306 carries nucleotide g that is the same as the reference sequence. Therefore, this change is not significant.

Although the four novel genes are strong potential candidates as hearing genes, based on the information on hand, they do not appear to be the causative gene(s) for hearing loss in this MSUDF1 family. Further information about their regulatory regions and the unidentified coding region will be needed before they can definitely be excluded.

DISCUSSION

Introduction

The hearing loss demonstrated by this mid-Michigan family (MSUDF1) is a novel form of nonsyndromic, genetic, late-onset, bilateral, progressive, sensorineural hearing loss. The affected family members report noticing the onset of bilateral hearing loss in the third decade of life, However, the hearing loss pattern, first evident at 6000 and 8000 Hz, can be detected using pure-tone audiology in some family members in their early teens and is clearly evident by the mid- to late-20s. As age increases, the degree of hearing loss increases with thresholds increasing at all frequencies, ultimately leading to a corner audiogram configuration (Elfenbein, et al. 2001).

Adult-onset sensorineural hearing loss is often attributed to presbycusis - the decline in hearing sensitivity caused by the aging processes - unless symptoms indicate the presence of a disorder such as otosclerosis. Although the onset of hearing loss associated with DFNA20 is earlier than would be expected with typical "age-related" hearing loss, the configuration and early pattern of progression is similar to that associated with the most common form of sensory presbycusis.

Intrinsic hearing loss decay/susceptibility to environmental factors

Presbycusis is thought to be due to a combination of genetic and environmental causes such as ototoxic drugs and noise exposure

(http://www.nidcd.nih.gov/health/pubs_hb/presbycusis.htm). These factors can be difficult to identify because of the time span over which environmental factors can have an impact. Thus, it is impossible to tell for sure whether the DFNA20 hearing loss has an intrinsic cause, such that the mutated gene itself causes disruption and/or death of the cells of the sensorineural apparatus, or whether the mutation merely causes particular sensitivity to environmental effect. However, the appearance of a steady progression with age when all affected family members are compared and the 1:1 ratio of affected to unaffected family members suggest that an intrinsic cause is most likely.

Functional candidates for the DFNA20 gene

Genes disrupted in hearing loss families are involved in various types of functions ranging from extracellular matrix, cytoskeletal, ion channel, synaptic vesicle trafficking to transcription factors (Table 1). Since a striking feature of the cochlea is its highly ordered architecture and the remarkable structural and spatial organization of the sensory hair cells themselves, cytoskeletal defects may also be particularly deleterious for auditory function. The gene(s) encoding inner ear specific ion channels, and components of the tip link and of the tectorial membrane will certainly represent good candidates for the DFNA20 gene.

The manner in which genetic conditions are inherited can sometimes give a clue to their function, however with regard to hearing loss genes; it has been observed that different mutations in a single gene can cause both recessive and dominant hearing loss, and

syndromic and nonsyndromic hearing loss. For example, the gene α-tectorin (TECTA) codes for a major component of the tectorial membrane (Legan, et al. 1997). The tectorial membrane is an acellular membrane that acts as a resonator and overlies the sensory hair cells of the cochlea. Mutations in α-tectorin (TECTA) can cause either dominant (DFNA8/12) or recessive (DFNB21) hearing loss (Verhoeven, et al. 1998, Alloisio, et al. 1999, Mustapha, et al. 1999). One unconventional myosin, MYO7A, expressed in the sensory epithelium of the inner ear is an actin-binding motor protein. Mutations in MYO7A cause dominant (Liu, et al. 1997a) and recessive (Weil, et al. 1997, Liu, et al. 1997b), nonsyndromic hearing loss and syndromic Usher's syndrome type 1B, a deafness –blindness disorder (Weil, et al. 1995).

Some genes implicated in hearing loss have unknown functions, for example, ICERE-1 gene is "inversely correlated with estrogen receptor expression" which is overexpressed in estrogen-receptor-negative breast carcinomas (Van Laer, et al. 1998, Thompson, et al. 1998). Its mutations were found in a single extended Dutch family with autosomal dominant sensorineural hearing loss (Van Camp, et al. 1995, Huizing, et al. 1966).

It is expected that many proteins have more than one function, depending on where they are found in the cell or within the body as a whole (Wolfsberg, et al. 2002). Recently the research group in University College London showed that a sugar transporter is a candidate for the outer hair cell motor (Geleoc, et al. 1999). The OHCs exhibit a voltage-dependent motility involving a 'motor' protein embedded in the basolateral membrane, which is important for normal hearing function.

Thus DFNA20 could be any of the genes that are important for potassium recycling and endolymph equilibrium, organelle transport, transcription or encoding cytoskeletal proteins, or structural proteins in the organ of Corti. Because hearing depends on a wide range of function, it is difficult to exclude a gene as a candidate for DFNA20 based on functional criteria. Any gene responsible for other diseases, both dominant and recessive, whether associated with hearing loss or not, for example, connexin 31 mutation for keratoderma, or for hearing loss or for hearing loss with keratoderma, is also a candidate gene for DFNA20. Genes with unknown function should not be ignored during the process of searching for DFNA20 gene.

In addition to the genes involved directly in the hearing loss there is evidence that some modifier genes can be involved in the hearing process. For example, DFNM1, a modifier (a dominant suppressor gene) of the profound hearing loss locus DFNB26 (4q31), is mapped to chromosome 1 (Wilcox, et al. 2001), within a region containing the DFNA7 locus (Fagerheim, et al. 1996). The dominant DFNM1 suppresses the homozygous profound congenital hearing loss in the DFNB26 families.

In this MSUDF1 family, we know that each family member who has the same haplotype shows a similar phenotype, therefore we surmise that they have the same mutation(s) in the same gene with full-penetrance. The possibility of having a modifier that suppresses the heterozygous DFNA20 gene(s) or interrupts the normal function of DFNA20 gene(s) is therefore not likely.

Possible types of mutation(s) of DFNA20 gene

Generally speaking, haploinsufficiency and dominant negative effect are the two major possible mechanisms involved in dysfunction of dominantly inherited mutated genes. Haploinsufficiency is a situation where reduction in the dosage of the gene product leads to phenotypic changes. It implies that the gene either has no product because of a deletion or the introduction of an early stop codon, or that the gene products - mRNA or polypeptide – are rendered so unstable as to be functionally severely reduced. The amount of product needed for normal function may differ in different cells, so that a range of phenotypes may be seen, depending on the level of functional gene product required. Dominant negative effects occur when the mutant product not only loses its own function or stability but also prevents the product of the normal allele from functioning in a heterozygous person. It is seen particularly when the gene product is required to interact with one or more polypepetides derived from the normal alleles or another gene. The mutation that causes a dominant negative effect is more likely to be one that results in a recognizable polypeptide with changes such as a single amino acid substitution or splicing change. At the gene level these would usually involve a single base change. There are a few examples of dominantly inherited genes in hearing loss families probably producing such a negative effect (DFNA2, DFNA8/12, DFNA11 and DFNA36) (http://www.uia.ac.be/dnalab/hhh/). Since the DFNA20 hearing loss is inherited in a dominant mode, the phenotype could be either due to haploinsufficiency or a dominant negative effect of the disease allele. If haploinsufficiency is the cause we

would expect to find deletion(s) or nonsense mutation(s) that disrupt the gene product (mRNA or polypeptide) completely, if a negative effect is the cause, we would expect mutations that produce a polypeptide product with abnormal function.

Possibility of more than one gene in the DFNA20 interval

In the DFNA2 locus, two hearing loss genes have been reported, GJB3 and KCNQ4, and it is possible that a third hearing loss gene is also at this locus (Goldstein, et al. 2002). If hearing loss in different families' maps to the same locus, two possibilities exist. Either they have the same or different mutations in the same gene or they have mutations in different genes that lie close together. If the same mutation in the same gene is common to two families and the mutation has arisen only once, the haplotype around the mutation in terms of SNPs and microsatellites is likely to be the same. If the mutations are different or the same mutation arose more than once, or if different genes are involved, the haplotype at the locus will more likely be different. Three families' map has been identified whose hearing loss maps to the 17q25 region, two DFNA 26 families and one DFNA20 family. The intervals for these families are overlapped with DFNA26 occupying the larger region. One of the two DFNA26 families and the DFNA20 family share a haplotype within the overlapped region (table 3). The possibility for them to have the same haplotype is about 20% considering the allele frequencies of these markers. So deafness in these two families is potentially caused by the same mutation of the same gene.

Physical candidates for DFNA20 gene

The DFNA20 interval was reduced to a region of approximately 1.5 megabases. From the data currently available, we can not tell for certain the exact number of genes in the defined locus, but on average, one should expect there to be approximately 20 genes in this interval. So far we have been able to construct a partial physical contig using additional markers based on the data from HGP. In the future, any gene responsible for nonsyndromic or syndromic, recessive or dominant hearing loss or for other diseases that map in the interval could be a candidate gene for DFNA20. Clearly those genes with functions similar to genes known to cause hearing loss or expressed in cochlea libraries would be first choice. However, if none of the obvious candidates turn out to be mutated, those genes without defined function that map in this interval must also be analyzed.

The reasons that DFNA20 gene has not been cloned

The genes responsible for hearing loss have not been identified at most of the hearing loss loci. Among 41 dominant hearing loss loci, only 17 genes have been cloned so far. This is due in part to the large intervals that can not be further refined because of limited family size and the markers available.

Identifying the genetic elements, whether intrinsic or predisposing, will be difficult without likely candidate genes. The great diversity of the possible causes of deafness can explain why it can be difficult to establish the causes of hearing loss in individual cases.

Examination of the mutation's effect on cochlear structure and function might help determine the gene involved, however the inaccessibility of the inner ear to discriminant diagnostic procedures make this impossible.

The draft human genome sequence covers about 94% of the human genome, but much work remains to be done to produce a finished sequence. The pericentromeric and subtelomeric regions of chromosomes contain large recent segmental duplications of sequence from elsewhere in the genome, and this duplication makes the sequence alignment difficult (International Human Genome Sequencing Consortium, 2001). The interval for DFNA20 is located on such a region, which is a possible reason for its not being completely sequenced. From our lab experience, using the end sequences of the screened BAC clones from the BAC library to search in the HTGS database, yields BACs from other chromosomes are also pulled out. This indicates that sequences in this interval may be duplicated elsewhere in the genome, which makes the correct sequence assembly difficult.

Cloning the genes responsible for isolated hearing loss in humans remains an important challenge. Due to the extreme genetic heterogeneity of this condition, the definition of a chromosomal interval for a deafness gene can only be based on the linkage analysis of large families with a clearly defined phenotype using polymorphic genetic markers. With the increasing sequence and expression data from the Human Genome Project and Celera, and the progress of mouse genome research, DFNA20 should eventually be cloned even though there is a lot of work to be done.

Future study

1. Fine mapping of the DFNA20 interval

The population living in Cornwall, England is stable and until recently was a remote group. Therefore, there is still a possibility that we can find individuals there who have a similar phenotype to MSUDF1 and who share a distant ancestor. Additional recombination that reduce the haplotype size and hence the locus interval might be found. As the sequence data from HGP and Celera are put in the public database, we should use the sequence data to look for new microsatellite and SNP markers, and type them in this family. Thus using the polymorphic markers and the extended relatives, it may be possible to refine DFNA20 critical interval.

2. Mouse models

There are many developmental similarities between humans and mouse ears. The mouse is frequently used as a model for hearing loss in human, by first finding the gene defect in mutant strains of deaf mice and then identifying the human gene through comparison of regions of homologous synteny between human and mouse chromosomes (Probst and Camper, 1999). The development of murine models of deafness have resulted in the rapid discovery of many disease loci, but there are still relatively few mouse models of specific forms of human deafness. A handful of human hearing genes corresponds with mouse models (http://ww.jax.org/research/hhim/documents/models.html)

(http://www.ihr.mrc.ac.uk/hereditary/mousemutants.htm). The gene that causes the jackson shaker phenotype maps to mouse chromosome 11 in a region with homology of synteny to human chromosome 17q25. The js mouse has now been excluded as a model for DFNA20, however a mouse model for the DFNA20 may be found or constructed. At present 90 percent of the mouse genomic sequence has been completed, the rapid progress in mouse genomic research could be very useful for identifying the DFNA20 gene. A murine deafness gene may be discovered that is a mouse model for the DFNA20 gene(s). Close attention should therefore be paid to the research progress in mouse genomics (http://hearing.bwh.harvard.edu/). Meanwhile, any mouse genes identified in a region on mouse chromosome that has homology of synteny to 17q25, but not identified in human should also be analyzed in this family.

2. Human Genome Project and other databases

Progress in the Human Genome Project (International Human Genome Sequencing Consortium, 2001) and availability of human cochlear-specific cDNA libraries (Robertson et al. 1994), cDNA libraries from auditory tissues of other organisms (Wilcox, et al. 1992, Heller, et al. 1998), the inner ear expression database from the Corey lab, and the gene expression database in the developing ear from the Steel lab (http://hearing.bwh.harvard.edu/) are now providing a repertoire of genes that are expressed in the cochlea. These provide powerful tools to identify candidate genes for deafness by a tissue–specific approach. To date, over 4000 cochlear expressed sequence tags (ESTs) have been sequenced from the human fetal cochlear cDNA library and

deposited in GenBank databases (Skvorak, et al. 1999). Analysis of these cochlear cDNAs provides a window on known and novel genes expressed in the cochlea (http://www.partners.org/pathology).

In addition to the databases for cochlear ESTs, there are EST databases from the Human Genome Project (http://www.ncbi.nlm.nih.gov/Basset/dbEST/PosiClonNew.html) that may be used to search ear-expressed genes. The I.M.A.G.E consortium provides a large repertoire of EST cDNA clones and their partial or full-length sequences (http://image.llnl.gov/). Some of the genes involved in the hearing process have been found using these ESTs databases. For example, using new polymorphic markers on the basis of genomic sequence information from the Washington University chromosome 7 sequence project, the DFNA5 region was refined to a region of 600-850 kb, where there are forty-three ESTs, positioned in the vicinity of DFNA5. The DFNA5 gene was found by characterizing these partial expressed cDNA sequences (Van Laer et al. 1998).

Based on the sequence data from HGP, the EST database, the human and other species cochlear cDNA database, any EST within the DFNA20 critical interval should be tested for the expression in the cochlea in either human or in mouse. Any cochlear EST should be treated as a strong candidate. By analysis of those ESTs, and use of the genomic sequences, the full-length cDNA sequence and intron-exon structure can be generated. The disease-causing mutations may be found by sequencing cDNA from the affected persons. Next, both alleles in the somatic hybrids should be sequenced, and we would need confirmation by looking for consegregation of the phenotype and the mutation.

3. Genes with known functions that are newly-mapped in this region

In the process of looking for the DFNA20 gene, there may be some newly identified genes in DFNA20 interval that are related to another disease(s). Any gene suspected to be involved in the hearing process will be potential candidate for DFNA20. Sequencing both alleles of the hybrid cell and typing the cosegregation of the genotype and the gene variation if any should be done.

Once a mutation is found in a gene that cosegregates with the phenotype, verification that it is the causative gene should be done in other families in which the hearing loss maps to the same region. These include the two DFNA26 families. Since the phenotype in this family is similar to presbycusis, except with earlier onset, the aging population with presbycusis should also be examined.

Finding the DFNA20 gene will help understand the process of normal hearing. In addition, identifying the DFNA20 gene may provide information about the genetic etiology of presbycusis, which is a common and disabling phenomenon of aging. It may eventually lead the way to specific prevention and treatment of auditory impairments due to either genetic or environmental causes.

Materials and Methods

PCR technique for standard and GC-rich region

The Polymerase Chain Reaction (PCR) process uses multiple cycles of template denaturation, primer annealing, and primer elongation to amplify DNA sequences (Saiki, et al., 1985). It is an exponential process since amplified products from the previous cycle serve as templates for the next cycle of amplification, making it a highly sensitive technique for the detection of nucleic acids. Typically, enough amplified product is generated after 20-30 cycles of PCR so that it can be visualized on an ethidium bromide-stained gel. For each pair of the newly designed primer pairs, I use a temperature gradient to determine the optimal annealing temperature (ranging from 56 – 67°C at 2 °C interval).

The typical reaction components were:

Components	**1 x (20µl)**
Template DNA (20-40 ng/µl)	1
sterilized, double distilled water	14.5
10 x PCR buffer (with 15 mM $MgCl_2$)	2
10 mM dNTP mix (Gibcol)	0.4
2.5 µM of each primer in primer pair mix	2
Thermostable DNA Polymerase (Gibcol or Perkin Elmer	0.1
Total	20

While most DNA fragments can be amplified by standard PCR methods. Some genes contain GC-rich regions that prevent their amplification by standard PCR techniques (Chenchick, et al., 1996). Because GC-rich sequences possess strong secondary structures that resist denaturation and prevent primer annealing, PCR often fails to yield any

product. In these cases, I used the CLONTECH's Advantage™-GC PCR Kit (cat. No. K1908-1).

The typical reaction components were:

Components	**1 x (25μl)**
Template DNA (20-40 ng/μl)	1
PCR grade water	6.9
5 x GC-genomic PCR buffer	5
25 mM $Mg(OAC)_2$	1.1
5 mM GC-melt	5
2.5 μM primer1	2.5
2.5 μM primer2	2.5
10 mM dNTP	0.5
Advantage GC polymerase Mix	0.5
Total	25

When no commercial GC-rich kit was available, DMSO was used instead. Frequently this produces the expected product, and fifty-eight degree centigrade was often chosen as my optimal annealing temperature. If it does not work well, a gradient was tested.

Components	**1 x (20 μl)**
Template DNA (20-40 ng/μl)	1
PCR grade water	10.5
10 x PCR buffer with 15 mM $MgCl_2$	2
2.5 μM primer1	2
2.5 μM primer2	2
10 mM dNTP mix	0.4
Thermostable DNA Polymerase (Gibcol or Perkin Elmer)	0.1
DMSO	2
Total	25

Table 12 lists the primers used to amplify each exon of the possible candidates on 17q25.

Table 12. Primers used in the initial analysis of the DFNA20 candidate genes.

	Primer Sequence	
Locus	**forward**	**reverse**
ACTG1	ggc agc act ttt att ttc cct	gtt act tgc ttt gag ttg gaa gc
ARHGDIA	agg aaa ggc gtc aag agt ga	ccc ata gct gcc tac cat gt
GRIN2C	ggc acc tac tga atg tca cct t	gag ggc gat cac cac ca
FKHL13	aga cca gtg tgt ctg tcc cc	aaa gtt gcc ttt gag ggg tt
P4HB*		
exon 1	cca ggc ccg gcg ctc ac	caa ggg agc cct tca gtt c
exon 2	gca tct att tcc gct tcc ag	cag gcc agt ccc tct cta aa
exon 3	tcc atg gtg gga gaa cat ct	gga aga ctg gaa tgc tct gg
exon 4	acc tct gct tct ccc tcc tc	gca gac caa ccc cag aac ta
exon 5**	agt cag ggc cct ctt tct gt	cag aag att ctc cca atg gc
exon 6	gtc tga cat tgg agg cca tt	aca ctt gtc acc tcg gga ag
exon 7	tgt gca acc tcc ttt tct cc	aca ctg aga gcc cag aga cc
exon 8	gga ttc ttg gca ctg ttg gt	agc tga agg gca aca ctt aca
exon 9	cca gtg gct ttg tgt cca g	ggg acc act gct ctt cca g
exon 10	ttc tct agg gag gag gag cc	gca cca ttc cca tca caa g
exon 11	ggt gtg agc tag tgt ggg gt	aga gag gtt ccc tgg gtt tc
D17S668	†	
D17S914	†	
D17S1806	†	
DNEL1		
exon 1	gtg agg agt tgg cat ctg ag	cta gag ggt tag ggt cca gt
exon 2	ttg cca ttc tgc ctc cac tt	agt cac ttc agg agt cct gc
exon3	cag gtt ggt tgg aac agt gg	ctc aca gag agc tgc att gg
exon4	ctt cgc agg taa gcac cac ta	tct taa cac ctg ctg tct gg
exon5	ctt cct tcc act cta ccc tc	ctt gaa ctc caa cag aga gc
exon6	ggg atg atc caa agc tct ag	tcc agt tgt cct gag tgg gc
exon7	gcc agt cct agc tct ata ac	agc aat gac cct gac ttc tg
exon8	ggt gat ctg agc gtgtgc at	gag aag cta aga gtg gat gg
exon9	tgg ctg cct tcc tga caa ct	cag ctg act gtg agg tgt gc
exon10	cac gaa ccc tga ttg taa cc	cta gac ccc tct gat tca cc
exon 11	ctc aat ctc aag aag cgat cc	ctg tct aag ccc ttt tgc ct
exon 12	gga tac tag ccc atg gac tc	tga cag gca gac atc tcc ag
exon 13	ctc gac aag cag gca aga gt	cta gga tgc tgc agg aca ac
exon 14	tgt cac cta ccc ctg gag tt	gat ggc ctc aag gaa aga ag
exon 15	aca aga gca agg ctg gtt gg	ggg ccc ttg tgg ttt gtt tc
GALR2		
exon 1	gca gag tcg cac tag gag tt	ctt gtc ttc cac tgc ctt ct
exon 2(1)	tcc tct gtg tgc ggt gta ac	cag gag ttg tcg tag gag ac
exon 2 (2)	cta ttc tgc ctc tgc tgg at	gct tcc tcc cag aat cta aa

** Primer pair used to type G3 and TNG RH panels for P4HB.

Preparation of DNA samples

Whole blood from Whole Blood Using the Gentra Kit (Gentra System, Inc. cat. no. D-T300) following the manufacture's instruction

DNA Preparation from swab sample

1. Swabs were collected in 5 ml STE solution.
2. Decant swabs and liquid into fresh 50ml tube (swabs now have tip up and plastic handle down), centrifuge 2000 rpm in 10 minutes.
3. Remove swabs from tube and transfer liquid to 15 ml orange capped polypropylene tube containing 5 ml phenol/$CHCl_3$/IAA (Isoamyl alcohol) (25:24:1), mix well.
4. Centrifuge 2000 rpm 5 minutes, using dispo transfer pipet, and transfer supernatant to 15ml tube containing 5 ml $CHCL_3$/IAA (24:1).
5. Centrifuge 2000 rpm 5 minutes.
6. Transfer supernatant to 15 ml tube with 5 ml 100% Isopropanol, invert to precipitate DNA, centrifuge 2500 rpm 10 minutes.
7. Add 70% Ethanol to wash the DNA pellet.
8. Drain pellets and allow to air dry.
9. Resuspend 300 µl puregene DNA Rehydration Solution overnight at room temperature.
10. Store at 4°C. For long term storage, place sample at –20°C or –80°C.

Microsatellite Genotyping

Two methods were used to genotype the markers used in this study: incorporating radioactively labeled nucleotides; or using fluorescent labeled primers. Guidelines for designing primers were used as following.

Look for regions with an even distribution of A, C, G, and T and put a G or C on the 3' end. Calculate the annealing temperature by giving 4 degrees for every G and C, and 2 degrees for every A and T, and try best to have an annealing temperature of round 60°C. If there is a lot of "N's" in the sequences covered, the primers were moved to a cleaner part of the sequence or pick another region. If strings of AAAAA or TTTTT are between genotyping primers, one expects to get a lot of stutter bands on the gel, therefore the primers were moved to the inside of these sequences.

The ABI PRISM™ 377 DNA Sequencer automatically analyzes DNA molecules labeled with multiple fluorescent dyes. After samples are loaded onto the system's vertical gel, they undergo electrophoresis, laser detection, and computer analysis, electrophoretic separation can be viewed on-screen in real-time, and final data can be output in a variety of formats, and final data can be output. GeneScan™ Size Standard are sets of fluorescent-labeled DNA fragments of known sizes used for determining the size of unknown DNA fragments run on ABI PRISM DNA™ sequenchers. The standards are run in the same lane as the samples, which contain fragments of unknown sizes labeled with different fluorophores. GeneScan ™ Analysis Software automatically calculates the size of the unknown DNA sample fragments by generating a calibration or sizing curve

based on the migration times of the known fragments in the standard. The unknown fragments are mapped onto the curve and the sample data is converted from migration times to fragment sizes.

Automated gel-based genotyping using microsatellites (fluorescent labeled pooling PCR reaction)

1. Setting up and running PCR: mix the reaction components listed in the followed table.

Reaction Components	**Volume (μl)**
Primer Mix (5 μM)	1.0
DNA (40-50ng/μl)	1.2
10 x PCR Buffer contain no Mg^{++}	1.5
dNTP mix (2.5 mM)	1.5
AmpliTaq®(5units/μl)	0.12
25mM $MgCl_2$	0.9
ddH_2O (PCR grade)	8.78
Total	15.0

2. Setting up the gel: making the 4.25% gel using special plates for the 377 instrument.

   a. Mix 18g urea, 5.3ml 40% acrylamide stock. 25ml water, and 0.5g of Amverlite MBI ionic exchange beads in a beaker.

   b. Stir without heat until the urea dissolves.

   c. Filter and degas the solution for 5 minutes.

   d. Add mixture to a graduate cylinder (100ml) and add 5ml of 10 x TBE. Dilute to the 50ml mark with water and mix solution.

   e. Before pouring, add 250 μl of 10% APS and 35 μl of TEMED.

   f. Pour gel, let polymerize for at least 1.5 hrs.

(Reagents must be fresh for these gels since they are thin and therefore more sensitive to reagent degradation effects.)

3. Pooling /loading the reactions (GeneScan):
   a. Pool the reactions in one plate (Pooling Plate), the ratios are 2 μl for HEX labeled samples, 1 μl for FAM, and 1 μl for TET. Make up the difference with water if the ratio is not 2:1:1. The formula is as follows:

      Final volume of HEX =Final volume (TET + FAM)

   b. Add 3 μl of loading cocktail to a plate (Loading Plate), the cocktail consists of 200 μl of deionized formamide, 40 μl of dextran blut/EDTA, and 40 μl of GS-350 size standard.
   c. Add 2 μl of DNA pool to the loading Plate. Set aside at 4°C until ready to load.
   d. If doing a sequencing reaction, only dissolve samples in formamide/EDTA according to the ABI kit protocol and set aside until ready to load. Samples can be stored safely in the loading buffer for only 2 hours.

4. Running the gel: Prerun the gel for 2-5 minutes. While pre-running, denature samples at 95°C for 5 minutes. Keep on ice for the rest of the experiment. Flush the sample wells with buffer in order to eliminate urea leakage. Load samples—2 μl for Gene Scan.

5. Post-gel procedure: Remove top buffer chamber and empty buffer first, then remove

bottom buffer chamber and empty buffer into sink, dry off any leaking liquid. Take apart plates, clean, rinse, and store in the holder.

6. Analysis: Genotyper 2.0 software was used to analyze the Gene Scan data files.

Manual gel-based genotyping using microsatellites (radioactive labeled PCR reaction)

1. Reagents

1mM dCTP: 10 µl of 10 mM dCTP + 90 µl H_2O

10 × (-dCTP):

Components	**100 µl**	**200 µl**	**400 µl**
10 mM dATP	20 µl	40 µl	80 µl
10 mM dGTP	20 µ l	40 µl	80 µl
10 mM dTTP	20 µl	40 µl	80 µl
1 mM dCTP	2.5 µl	5 µl	10 µl
ds H_2O	37.5 µl	75 µl	150 µl

Stored up to 6 months at –80°C.

Deionization of formamide: 500 ml of formamide was mixed with 50 g ion exchanger (AG 501 –X8 Resin) from Biorad, stir 30 min slowly on a stirrer, then remove resin by filtration and store the deionized formamide at -20°C

Gel loading buffer: add 10 ml deionized formamide, 10 mg xylene cyanol FF, 10 m g bromophenol blue, 200µl 0.5 M EDTA, mix, stored in a dark place or wrapped with aluminum foil at room temperature

Gel slick™ solution: Cat. No 50640 (250 ml), stored at 18 ~ 26°C

Sequagel system: Sequencing system Diluent (Life Scientific Product. Ltd. order no. EC-840) 450 ml / 1 liter, sequencing system Concentrate (Life Scientific Product. Ltd. order no EC-830) 450 ml / 1 liter and sequencing system buffer (Life Scientific Product. Ltd. order no. EC-835) 100 ml / 1 liter

2. Radioactive PCR Recipe:

Components	1 x (15) µl
H_2O	10.055
10 x PCR buffer (PE with 15 mM mg^{++})	1.5
10 x (-dCTP)	0.75
10 x primer pair (2.5 µM)	1.5
α-^{33}P-dCTP (10µci/µl)	0.105 (0.07 µl/10 µl Rnx)
PE AmpliTag (5 U/ µl)	0.09
Genomic DNA template	1

If the amplified marker region is GC rich region, follow the recipe is described as below.

Using advantage®-GC Genomic PCR Kit (CLONTECH cat. no. K1908-1)

Components	1 x (15 µl)
PCR grade H_2O	2.985
5 x GC Genomic PCR buffer	3
25 mM $Mg(OAC)_2$	0.66
GC-melt (5M)	3
2.5 µM primer1	1.5
2.5 µM primer2	1.5
10 x (-dCTP)	0.75
α-^{33}P-dCTP ((10µci/µl)	0.105
Advantage GC Polymerase mix	0.5
Genomic DNA template	1

1. Setting up the gel

Making acrylamide gel

Component	**100 ml**	**50 ml**
SequaGel concentrate	24 ml	12 ml
SequaGel diluent	66 ml	33 ml
SequaGel Buffer	10 ml	5 ml
10 %APS (Ammonium Persulfide)	500 µl	250 µl

Swirl gently to mix and filter it by 0.45 µm filter, add 20 µl TEMED, swirl gently, pour gel, let it polymerize at least 2 hrs.

10% APS (1g APS + 10 ml H_2O) should be fresh weekly. The glass plate should be coated with Gel slick source and cleaned with ethanol before pouring gel.

2. Preparing the reactions for loading

   Add equal volume of loading buffer to the labeled reaction. The reaction was heated at 95°C for 5-10 min, and set in the ice water immediately to keep the labeled PCR product denatured.

3. Running the gel: Prerun the gel for half an hour or until the gel is heated up to above 40°C. Load the 5 – 8 µl of the denatured reaction, loading a gel should not take too long in order to avoid the gel temperature falling down below 40°C. Run the gel at an appropriate voltage to keep the gel at 40°C all the running time. Usually the running time is about 1-2 hours, but it really depends on the size of the markers.

4. Post-gel procedure: Peel the gel off the glass plates and put it onto the gel drier, 80°C for about 2 hours or until the gel is completely dry. Expose the dried gel to X-ray film

overnight, if the signal is too strong with lots of Cytidine labeled, exposure should be reduced to several hours. The plates were cleaned with biologic soap right away.

5. Analysis: Analysis for manual genotyping takes a lot of practice. Some markers are prettier than others and it is really easy to score their alleles. Other markers have a lot of stutter bands, which make things more difficult. After scoring the genotypes were entered by hand into the computer for linkage analysis or put onto the family pedigree for later haplotype analysis.

Table 13 lists the primers for the microsatellites used in this project.

Table 13. The microsatellite markers I typed in MSUDF1 family.

Primer Kf #	Length(bp)	Type	BAC	Annealing T.(°C)	Comments
Kf 640/641	208	tetra	31B8	61	informative
Kf672/673	193	di	497H17	63	not score well
Kf674/674	198	tetra	497H17	63	uninformative
Kf642/643	205	di	17J14	58	ignore
Kf644/645	182	di	17J14	58	ignore
Kf680/681	226	di	497H17	58	not score well
Kf682/683	203	di	497H17	58	informative
Kf684/685	218	di	497H17	58	informative
Kf699/700	188	penta	455O6	56 (GC-rich)	informative
Kf701/702	192	tetra	455O6	58 (GC-rich)	uninformative
Kf703/704	203	tetra	455O6	59	uninformative
Kf705/706	207	penta	455O6	58 (GC-rich)	uninformative
Kf707/708	206	penta	498C9	57	uninformative
Kf709/710	203	di	498C9	55	uninformative
Kf711/712	201	tri	455O6	63	not score well
Kf713/714	206	di	455O6	63	uninformative
Kf715/716	195	di	455O6	63	informative
Kf717/718	165	penta	498C9	56	not score well
Kf719/720	195	di	98G11	56	uninformative
Kf721/722	197	di	98G11	56	not score well
Kf723/724	201	di	98G11	not work	
Kf725/726	196	di	98G11	61	not score well
Kf727/728	191	di	334C17	61	informative
Kf729/730	197	di	334C17	53	informative
Kf731/732	189	tetra	334C17	61	informative
Kf733/734	185	di	334C17		informative
Kf738/739	D17S761		31B8	61	uninformative
Kf741/742	D17S1168		31B8	56	not score well
Rob I			474I11	61	uninformative
Rob II			474I11	58	informative
Rob III			474I11	61	uninformative
Kf768/769	163	di	28G8	55	informative
Kf770/771	184	tri	313F15	55	not score well
Kf772/773	182	di	313F15	55	informative
Kf774/775	164	di	810O4	55	informative
Kf776/777	237	tri	98G11	60	uninformative
Kf778/779	237	tetra	98G11	60	uninformative

Kf781/782	209	tetra	98G11	58	uninformative
Kf783/784	201	di	98G11	58	not score well
Kf785/786	230	di	31B8	58	informative
Kf787/788	119	tri	31B8	58	uninformative
Kf789/790	170	di	498C9	63	uninformative
Kf791/792	168	tetra	498C9	63	informative
Kf797/798	255	di	98G11	not amplified	
Kf799/800	194	di	98G11	58	not done
Kf801/802	300	di	98G11	58	not done
Kf803/804	360	tetra	98G11	not amplified	
Kf805/806	197	tetra	498C9		not done
Kf807/808	241	di	498C9		not done
Kf809/810	245	di	405A4	62	not score well
Kf811/812	194	di	405A4	54	Informative
Kf819/820			353N14		not done
Kf821/822			353N14		not done
Kf824/825	D17S784		98G11/353N14	61	informative
Kf826/827	D17S928		516M14	61	informative
Kf962/963	199	di	494E11	not amplified	
Kf964/965	175	di	494E11	55	informative
Kf966/967	126	di	494E11	55	informative

SNPs genotyping (Single nucleotide polymorphism)

SNPs represents the newest highly automated genotyping technique. Changes in nucleotide sequence by one base substitution represents the most common genetic variation, so SNPs are highly abundant, more frequent than microsatellite markers. These polymorphisms caused by a single nucleotide change are not a repeat, this type of polymorphism can be detected through either sequencing directly, a restriction digest, or by SSCP.

1. Design primer pair to amplify the region that has a SNP, if the SNP creates or blocks a restriction site, choose an appropriate enzyme to digest the PCR product. If not, do direct sequencing for each individual.
2. Score the allele by following the family pedigree to find out the linkage or heredity. SNP alleles are not so polymorphic as the microsatellites, as they are only two alleles.

RH Assay Protocol

The following is a description of the protocol used in the NIH to assay for linkage of STSs or genes for radiation hybrid mapping.

Radiation hybrid (RH) mapping makes use of a panel of somatic cell hybrids, with each cell line containing a random set of fragments of irradiated human genomic DNA in a

hamster background. In this study, two RH panels were used: the Stanford TNG4 panel and G3 panel. DNA from each of the cell lines in these panels has been prepared in large batches and is readily available.

	Stanford (TNG4)	**Stanford (G3)**
X-Ray dosage	50,000 rad	10,000 rad
Number of hybrids in mapping panel	90	83
Relationship of X-ray breakage to distance,1% breakage	=4kb	= 25kb
Average size of human fragments	800kb	4 Mb
Average resolution of comprehensive map	60kb	267kb
Average resolution of 1000:1 map	100kb	1.4Mb

An STS marker or a gene is typed against an RH panel by using its pair of oligonucleotide primers to perform a PCR assay against the DNA from each hybrid cell line of the panel. The results are recorded as a vector of 1's and 0's to indicate presence or absence of the STS or gene in each hybrid. Each STS or gene will give a pattern of retention for a given panel. These retention patterns can be compared to each other. Very similar retention patterns have high lod scores.

Two very similar retention patterns can be explained by very few breaks induced by radiation between the two STSs or genes such that they are lost and retained together in the hybrid panel. Given random X-ray induced breakage, STSs which are close to each other in the original human genome would have fewer breakage between each other than STSs which are further apart therefore similar retention patterns indicates close STSs and generate high lod scores. The distance measure between two STSs, "cR" is a measure of the amount of breakage between two STSs. The likelihood of a break per physical

distance unit (such as kb) goes up as the radiation dose increases. Therefore the distance between two STSs must be indicated by a measure, which indicates the radiation dose such as cR10,000 (for the G3 panel), or $cR_{50,000}$ (for the TNG4 panel).

Constructing a map from these data requires computational analysis of the RH vectors. Two markers are considered to be linked if they have vectors of statistically significant similarity (defined by a LOD score), and a measure of their separation is obtained from analysis of the degree of difference between the two vectors. Totally linked markers have identical scores, and therefore cannot be resolved by the RH panel used. If a sufficient number of markers are typed on one panel, then continuous linkage is established along each arm of a chromosome, and the markers are assembled into the map as a single linkage group.

Two RH panels were used to decide which of the candidates were inside or outside of the DFNA20 critical region. The 1's and 0's data we typed were sent thorough to Stanford in order to use their computational analysis of the RH vectors. The result about the linkage and RH distance was returned in a couple of days.

5' RACE (Rapid Amplificaion of cDNA Ends) was performed according to the kit manufacturer's instruction GIBCOBRL kit (cat. no. 18374-058)

Screening of BAC library

The human BAC (Bacterial Artificial Chromosome) Library used for this project is from Research Genetics (Catalog No. 96011). This BAC library pools are supplied as PCR ready plasmid DNA arranged in an easy to screen format. Human BAC plasmid DNA pools were provided in 96-well microtiter plates: One Superpool plate, and four Plate Pool plates, and 24 Row/Column plates. Each Superpool consists of plasmid DNA prepared from arrayed clones from eight 384-well microtiter Library plates. The Human BAC Personal Size Release is a subset of the Human BAC DNA Pools Release IV. As such, it includes only Superpools 25-72, corresponding Plate Pools 193-576, and Row and Column plate numbers 13-36.

Each well of the Superpool plate consists of plasmid DNA prepared from arrayed clones from eight 384-well microtiter Library plates. Superpool #1 consists of plates 1-8; superpool #2 consists of plates 9-16, and so on. There are a total of 96 superpools. The superpools are placed in the 96-well microtiter plate.

Each of the DNA pools in the plate labeled "Superpool" was tested initially, There will be 96 or 48 PCR reactions (if Personal Size Release) in this step. A positive well represents eight plates. Next, use the Plate Pool Screening Guide provided by Research Genetics to determine which plate(s) to test based on the results of the Superpool

screening. Set up a PCR for each plate represented in the positive Superpool(s). The Row/Column pools associated with the Plate Pool may be set up at the same time. The appropriate set of Row/Column pools were located by finding the Row/Column Pool label that lists the set of eight Library Plates, which are being screened at the Plate Pool level. To avoid cross-contamination of adjoining wells, spin the plates briefly in a centrifuge at a very low RPM before use. The plates were used without removing the adhesive seal by poking a hole through the seal above the well(s) to use. The holes can then be resealed with cellophane adhesive tape. Removing the adhesive seal is not recommended.

PCR conditions will vary according to primer length and base composition, reaction volume, and the type of temperature cycler used.

Screen of Superpool of RP-11 BAC library

1. Thaw the superpool plate, centrifuge at 15,000 rpm, 20 second with plate rotor.
2. Take 11 μl from each well to the round bottom well plate.
3. Add 44 μl H^2O to each well (the dilution factor is 1:5), mix them with 8 channel pippette.
4. Aliquot 5 μl to each well of the working plate, totally the 55 μl can be distributed to 10 working plates. MicroAmp opti-well conical plates do not have lids, so cover with strip caps, stored at –20°C.

Plate Pools

A plate pool is simply a plasmid preparation of all the clones from a single 384-well microtiter library plate. There are 768 plate pools arrayed into eight 96-well microtiter plates. Each column of the 96-well plates represents the eight plate pools contained in one superpool. One positive corresponding to each superpool positive should be obtained at this level.

Row and Column Pools

Row and column pools are made by combining all of the like rows or columns of each set of eight library plates which make up each superpool. Each Row and Column Plate contains two sets of Row and Column pools: one set on the left –hand, and one set on the right-hand side. The correct row and column pools are located in the wells directly under the label. Two positives at this level were obtained (one for a row designation, and one for a column designation).

Standardized clone names

A clone is identified by its microtiter plate address (plate number, row, and column) when you screen the library. For example RPC11-442p22 (Library-Plate, row. Column). Using this identified clone address, human BAC clone were ordered from Research Genetics.

Following is a rapid alkaline lysis miniprep method for isolating DNA from large BAC clones quoted from the kit provided by the Research Genetics.

Solutions:

P1 (filter sterilized, 4°C)

15mM Tris, pH 8.0

10 mM EDTA

100 ug/ml RNase A

P2 (filter sterilized, room temp)

0.2N NaOH

1% SDS

P3 (autoclaved, 4oC)

3M KOAc, pH 5.5

Procedure:

1. Using a sterile toothpick, inoculate a single isolated bacterial colony into 2 ml TB (or LB) media supplemented with 25μg/ml chloramphenicol (for BACs). Use a 12-15 ml snap-cap polypropylene tube. Grow overnight (up to 16 h) shaking at 225-300 rpm at 37°C.
2. Remove toothpicks using forceps. Centrifuge (SM24 or similar rotor) at 3,000 rpm for 10 min. of spin in the Sorvall. The temperature of the spin is not critical at this stage.

3. Discard supernatants. Resuspend (vortex) each pellet in 0.3 ml P1 solution. Add 0.3 ml of P2 solution and gently shake tube to mix the contents. Let sit at room temperature for 5 min or so. The appearance of the suspension should change from very turbid to almost translucent.
4. Slowly add 0.3 ml P3 solution to each tube and gently shake during addition. A thick white precipitate of protein and E. coli DNA will form. After adding P3 solution to every tube, place the tubes on ice for at least 5min.
5. Place tubes in the SM24 rotor and spin at 10,000 rpm for 10 min at 4 °C.
6. Remove tubes from centrifuge and place on ice. Transfer supernatant using a P1000 or a disposable pipette to a 1.5 ml eppendorf tube that contains 0.8 ml ice-cold isopropanol. Try to avoid any white precipitate material. Mix by inverting tube a few times; place tubes on ice for at least 5 min. At this stage, samples can be left at -20 C overnight.
7. Spin in cold microfuge for 15 min.
8. Remove supernatant and add 0.5 ml of 70% EtOH to each tube. Invert tubes several times to wash the DNA pellets. Spin in cold microfuge for 5 min. Optional --repeat step 8.
9. Remove as much of the supernatant as possible. Occasionally, pellets will become dislodged from the tube so it is better to carefully aspirate off the supernatant rather than pour it off.
10. Air dry pellets at room temp. When the DNA pellets turn from while to translucent in appearance, i.e., when most of the ethanol has evaporated, resuspend each in 40 ul TE. Do not use a narrow bore pipets tip to mechanically resuspend DNA sample;

rather, allow the solution to sit in the tube with occasional tapping of the bottom of the tube. For large PAC clones resuspension may take over 1 hour.

11. Use 5 ul for digestion with NotI or other rare cutter enzymes. There are NotI sites flanking the Sp6 and T7 promotor regions of the pBeloBAC11 vector; therefore, this is a very useful enzyme for analysis of insert size and for partial digest restriction mapping. Use 7-10 ul for digestion with a more frequent cutter such as BamHI or EcoRI.

Preparation of DNA Template for Direct Sequencing of Large Insert BAC Plasmids modified according to the manufacturer's instruction (QIAGEN® Plasmid Midi Kit)

(This procedure has been used to isolate 150-250 kb BAC DNA of a human-BAC library cloned in pBeloBAC11 from Escherichia coli strain HB101/r. The yield of BAC DNA from 100ml culture was 20 – 40 μg.)

1. Streak clone stock to single colony on LB plate containing 25μg/ml of chloramphenicol.
2. Pick a single BAC colony and inoculate a starter culture of 5ml LB medium (+appropriate antibiotic such as chloramphenicol), grow overnight at 37°C.
3. Inoculate with 0.5 ml pre-culture into 100ml selective LB medium. Grow at 37°C for 14 hours with vigorous shaking (~250 rpm).
4. Divide the cells into two 50-ml tubes, and harvest the cells by centrifugation at 45,000x g for 20 min.

5. Resuspend each bacterial pellet in 10 ml Buffer P1 containing 100μg/ml of RNase A.
6. Add 10 ml Buffer P2 to each tube. Mix thoroughly and gently by inverting 4-6 times, and incubate at room temperature for 5 min.
7. Add 10 ml chilled Buffer P3 to each tube. Immediately mix by gently inverting 4-6 times, and incubate on ice for 15 min.
8. Centrifuge at ≥ 20,000 x g for 30 min at 4°C. Remove supernatant containing plasmid DNA promptly.
9. Re-centrifuge at ≥ 20,000 x g for 30 min at 4°C. Remove supernatant containing plasmid DNA promptly.
10. Equilibrate a QIAGEN-tip 100 by applying 4 ml Buffer QBT, and allow the column to empty by gravity flow.
11. Pool the two supernatants from step 8. Apply the sample to the QIAGEN-tip and allow it to enter the resin by gravity flow.
12. Wash the QIAGEN-tip with 2 x 10 ml Buffer QC.
13. Elute DNA with 5 x 1 ml Buffer QF, pre-warmed to 65°C.
14. Precipitate DNA by adding 3.5 ml room-temperature isopropanol to the eluted DNA. Mix and centrifuge immediate at ≥ 15,000 x g for 30 min at 4°C. Carefully decant the supernatant.
15. Wash the DNA pellet with 2 ml of room temperature 70% ethanol and centrifuge at ≥ 15,000 x g for 10 min. carefully decant the supernatant without disturbing the pellet.
16. Air-dry the pellet for 5-10 min, and redissolved the DNA in a suitable volume of buffer or water for sequencing.

Phage library Screening

The phage library screening technique used was a PCR-based method (Israel, 1993). Initially, a single colony of host cell, XL1-Blue-MRF Kan, was inoculated into 25 ml of LB medium with kanamycin (0.75 mg/ml), cultured at 37°C overnight. Harvest the cells at 2000 rpm, 10min. Appropriate amount of 10 mM $MgSO_4$ was added to suspend the cell pellet, and read OD_{600}, then the cell suspension was diluted to OD_{600} of 0.5.

Phage library was diluted 1:100 with SM buffer (approximately 5 x 10^8 pfu/ml). 5µl of diluted phage infected 2.4 ml of host cell at 0.5 of OD_{600}, 15 min at 37°C to allow phage to attach the cells. The infected phage culture was divided into 64 wells and grown until the culture became cloud, then clear, 15µl of phage culture from each of the 8 wells across columns and 15µl of phage culture from each of the 8 wells across rows were pooled. Each pool contains 120µl (15µl x 8), 50µl from column and row pool were pipetted out, equal volume of 100mM NaOH was added to the column and row aliquots, boiled at 95 °C, 10 min, then 10µl of 1M Tris•HCL (pH 7.4) was added and mixed thoroughly. These treated phage pools were screened for the STS of interest by PCR. A single well that contained the known sequence was founded. This gave an address with column and row path. This well was then subdivided into 64 wells, each containing approximately 30 individual phage, the screening procedure was repeated. A positive well was then identified. This positive well was diluted and subdivided into 64 wells, a third round of screening was done, a positive well was finally identified, which contained one single phage clone that has the STS of interest.

The positive phage clone was grown, total phage DNA was extracted and sent for sequencing.

Total phage DNA preparation

10 x TM buffer: 100 mM Tris.Cl, pH 8.0

100 mM $MgCl_2$

1. Add 8 ml TM buffer and 8 ml phage lysate to a 30ml polycarbonate centrifuge tube. Add 32 µl of 10 mg/ml DNase I to degrade bacterial DNA.
2. Add 1.6 ml 5 M NaCl, 1.8g PEG 8000 (final concentration would be 0.5M NaCl, and 11.25% of PEG 8000) to precipitate phage particle. Mix well, PEG must be completely dissolved. Put on ice > 1 hr.
3. Centrifuge 10,000 x g, 20 min.
4. Resuspend in 500 µl TM and transfer to a 1.5 ml microfuge tube.
5. Add 500 µl of $CHCl_3$, vortex well, microfuge 5 min, transfer supernatants to fresh 1.5 ml microfuge tube.
6. Add 25 µl of 0.5 M EDTA (PH 8.0), 50 µl of 5.0 M NaCl 500 µl of phenol, vortex well, microfuge 5 min. transfer supernants to fresh 1.5 µl microfuge tube.
7. Extract supernatant with 500 µl of $CHCl_3$, Transfer supernants to screwed capped microfuge tubes. Add 1 ml EtOH. Precipitate DNA in dry ice/ethanol bath for 10 – 15 min or at –20°C for several hours or overnight.

8. Microfuge DNA for 15 min. Decant supernatnats and dry pellets for 10-15 min in vacuum desiccator. Resuspend in 50 µl TE. 10 µl should yield enough DNA to be visible on a medium sized agarose.
9. Add RNase A to the suspended phage DNA solution, incubate at 37 °C for 30 min to remove RNA or at the first step, add 1 ml of 40 mg/ml RNase A solution.

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APPENDIX

A Mitochondrial Mutation Associated with Nonsyndromic Sensorineural Hearing Loss

Summary

Maternally transmitted non-syndromic sensorineural hearing loss was described recently to be associated with mitochondrial DNA (mtDNA) mutations. I report here that a mutation in the mitochondrial $tRNA^{ser}$ (UCN) gene at position 7445 associated with maternally inherited sensorineural hearing loss. This mutation shows heteroplasmy in DNA from peripheral DNA and buccal sample DNA. To examine whether heteroplasmy levels in peripheral blood DNA change upon onset and severity of hearing loss. Quantitations of mutant mtDNA were determined in DNA samples from peripheral blood, collected recently from 25 individuals with the 7445 mutation with deafness or not. The results clearly demonstrate the discordance between the degree of heteroplasmy and the degree of hearing loss.

Introduction

Human mitochondrial DNA (mtDNA) is a 16,569 nucleotide pair (np) closed circular molecule, which provides 13 polypeptides, each of which requires mitochondrially encoded 12s and 16s rRNA and 22 transfer RNAs (tRNA) for their translation (Wallace, 1992; Anderson et al., 1981.). The cytoplasm of eukaryotic cells contains hundreds of mitochondria and thousands of mtDNA. The only known function of the human

mitochondrial chromosomes is the production of energy in the form of ATP, which occurs through oxidative phosphorylation in the inner membrane of the mitochondrion (Veltri et al., 1990). These mt chromosomes are all maternally derived, and in the normal individual are considered to be all identical ('homoplasmy'). In most known mutated mitochondria, normal and mutated chromosomes coexist in the same cell ('heteroplasmy').

MtDNA is particularly prone to acquire mutations for two reasons: (1) mt DNA is exposed to high concentrations of free oxygen radicals generated by the respiratory chain, particularly in tissues with a high-energy requirement. Oxygen radicals produce nicks in the DNA phosphodiester backbone, which could promote deletion during DNA replication, and oxidize bases, which can lead to the misincorporation of specific nucleotides during replication. (2) mtDNA lacks both a histone coat and at least some of the DNA repair enzymes, which are protective nuclear mechanisms against unfaithful replication .

Since the initial discovery of alterations in mitochondrial DNA as pathogenic factor (Holt et al, 1988; Wallace et al, 1988), numerous additional mtDNA mutations have been reported to associate with a variety of diseases that mostly affect human organs such as neural system, muscular system and auditory system whose functions are dependent on miochondrial oxidative phosphorylation process (OXPHOS).

So far, a number of types of mitochondrial mutation have been found associated with non-syndrommic hearing loss, or with the syndrome of diabetes and hearing loss. These

include large heteroplasmic re-arrangement (Bakkinger et al., 1992; Dunbar et al., 1993), heteroplasmic point mutations in tRNAs (Van Den Ouweland JMW et al., 1992; Reardpm W, 1992; Reid FM, 1994) and a homoplasmic mutation in the 12s rRNA gene (prezant, 1993). Here I describe a large maternal lineage with nonsyndromic sensorineural hearing loss, harboring a mitochondrial mutation in the $tRNA^{ser}$ (UCN) gene, at np7445. In some members of the lineage the mutation is heteroplasmic at a low level they have hearing loss, and one member who has homoplasmic mutation has normal hearing, within the limits of detection by PCR-RFLP analysis and direct sequencing of PCR products.

In order to learn more about the features of this disorder, and to determine whether its severity is correlated with the degree of heteroplasmy in blood, we carried out pure-tone autiometry on affected and unaffected member of the family, and compared the results with the representation of mutant mtDNA in the individual surveyed. The results show that the severity and age of onset of the disorder are not closely correlated with the apparent amount of heteroplasmy in blood, and are consistent with the apparent amount of heteroplasmy in causative factor, whether nuclear genetic or environmental, remains to be identified.

Materials, Patients, and Methods

Family Data

The large pedigree with nonsyndromic sensorineural hearing loss is shown in Figure 1. The details of the pedigree structure have been obtained. auditory examination and formal genetic analyses were performed. The deafness was transmitted exclusively through the maternal line, but in some cases through unaffected females.

Audiometry

Pure-tone audiometry was performed in an acoustically treated booth, one of these tests is shown in Figure 2.

DNA Preparation and PCR

Blood samples (5ml) were collected into heparin tubes, and either processed immediately to extract DNA or white blood cells stored at -20^0 C, DNA was prepared by using DNA isolation kit by Genetra System. 2μl of this was used in a 25μl PCR reaction. Oligonucleotide primers (np 7178-7198 and np 7840-7821) of human mt DNA; Anderson et al., 1981) were synthesized using an Applied Biosystems DNA synthesizer. Blood DNA was amplified using these primers, resulting in a 662 bp product spanning the 3'

end of the mitochondrial COI gene, the entire genes for $tRNA^{ser}$(UCN) and $tRNA^{asp}$, and the 5' end f the COII gene.

PCR condition for 30 cycles were 30 sec at 94^0C, 30 sec at 58^0, 1 min at 72^0C.

Restriction Enzyme Analysis

The PCR products were digested with 10 units of xbaI (GIBCOBRL), and then the digested PCR product eletrophoreses through 3% NuSieve agarose gel.

DNA Sequencing

Four replicate PCR reactions were set up for the blood DNA samples of the proband's mother, the four reaction products were pooled and purified with Wizard™ PCR Preps DNA Purification System (Premega) before sequencing. After quantitation of purified PCR products in terms of standard Mark V (GIBCOBTL), the fluorescently-labelled dideoxynucleotides were used for our further sequencing using ABI373 DNA Sequencer, sequencing primers used were np7178-7198 and np 7840-7821 of the human mtDNA sequence (Anderson et al., 1981).

Quantitation of Mutant MtDNA

To estimate mtDNA damage and evaluate the correlation between the degree of mutant mtDNA and the severity and age-onset, the proportion of the total mtDNA harboring the mtDNA mutation at np7445 was determined.

First DNAs of known homoplasmic mutant mtDNA and normal mtDNA were serially diluted and these dilutions were used for PCR amplification across the point mutation, at np7445. Posterior to amplification, the PCR samples was digested with xbaI, which cut normal mtDNAs and uncut the mutant mtDNA, at np7445. 4μl of digested PCR products was electrophoresed on the same 3% NuSieve agarose gel containing ethidium bromide, photographed under UV translumination and analyzed by densitometry (Shoffner et al, 1990).

The standard curve with the percentage of mutant at y-axis and the logrithm of ratio of mutant over normal mtDNA at x-axis was used to correct the heterodimer formation which results in an underestimation of the degree of mutated DNA after xbaI cleavage (Larsson et al., 1992).

Using the standard curve, we estimated the percentage of mutant mtDNA for the family members of this maternally inherited pedigree.

Results and Discussion

The pedigree of the family under investigation is given in Figure 1. Seven members of this pedigree are known to suffer from nonsyndromic sensorineural hearing loss, of varying severity and age-onset. The pedigree shows that there is no instance of inheritance via the paternal line. In seven branch of the family, the trait appears to have been passed on via the maternal line including one individual who herself is unaffected, but she has affected children. Most cases of apparent maternal nontransmission are children, for whom it is too early to draw a definite conclusion. The pedigree is strongly suggestive of mitochondrial transmission.

Figure 3 shows the results of xbaI digestion of PCR products spanning the junction of the COI and $tRNA^{ser}$ (UCN) genes. As shown, the amplified products from the control, the married-in members were cut, which resulted in formation of two bands, 395bp and 267bp, while those from family members of the pedigree were not, which resulted in one 662bp band.

At the bottom of the Figure 3 is shows the percentage of mutant mtDNA of each member of the partial pedigree indicated on the top of the gel image. As show in Figure 3, some affected members including the proband and his mother are homoplasmic mutation in $tRNA^{ser}$ gene at np7445, others are heteroplasmic (coexistence of normal and mutant mitochondria in the same cell), one unaffected member is homoplasmic mutation, others are heteroplasmic.

Comparison of audiometry with the percentage of mutant mtDNA shows clearly the discordance between the degree of heteroplasmy and the degree of hearing loss. The 7445 mutation is a silent change of both the last nucleatide of the cytochrome oxidase I gene on the heavy strand and the nucleotide immediately adjacent to the 3' end of he $tRNA^{ser}$(UCN) gene on the light strand. Mechanistically, the mutation appears to interfere with normal processing of the light-strand polycistronic mRNA. Significant decreases both in the amount of $tRNA^{ser}$(UCN) and in the rates of mitochondrial protein synthesis have been observed (Guan et al., 1997). The following explanations could be advanced for the incomplete penetrance observed. First, the tissue-specific expression of certain mitochondrial genes results in differential accumulation of mtDNA damage in somatic tissues, and the mitochondrial mutation could interfere with a tissue-specific secondary function of the mitochondrial gene, which also makes the cell more sensitive to changes in oxidateve-phosphorylation capacity. Second, a combination of nuclear factor and/or other mitochondrial alteration complicates the penetrance of this disorder. Third, environmental factors experienced by different members of this pedigree modify the expression of the mutated gene.

Thus the following questions remain to be answered: why does the same mutation cause severe hearing loss in some family members but not in others, and why is the ear the only organ affected?

Figure 1. Pedigree of family with sensorineural deafness of mitochondrial transmission. Subjects are tested by audiometry. The arrow points proband.

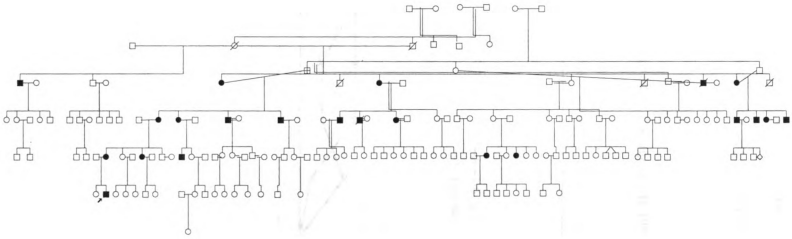
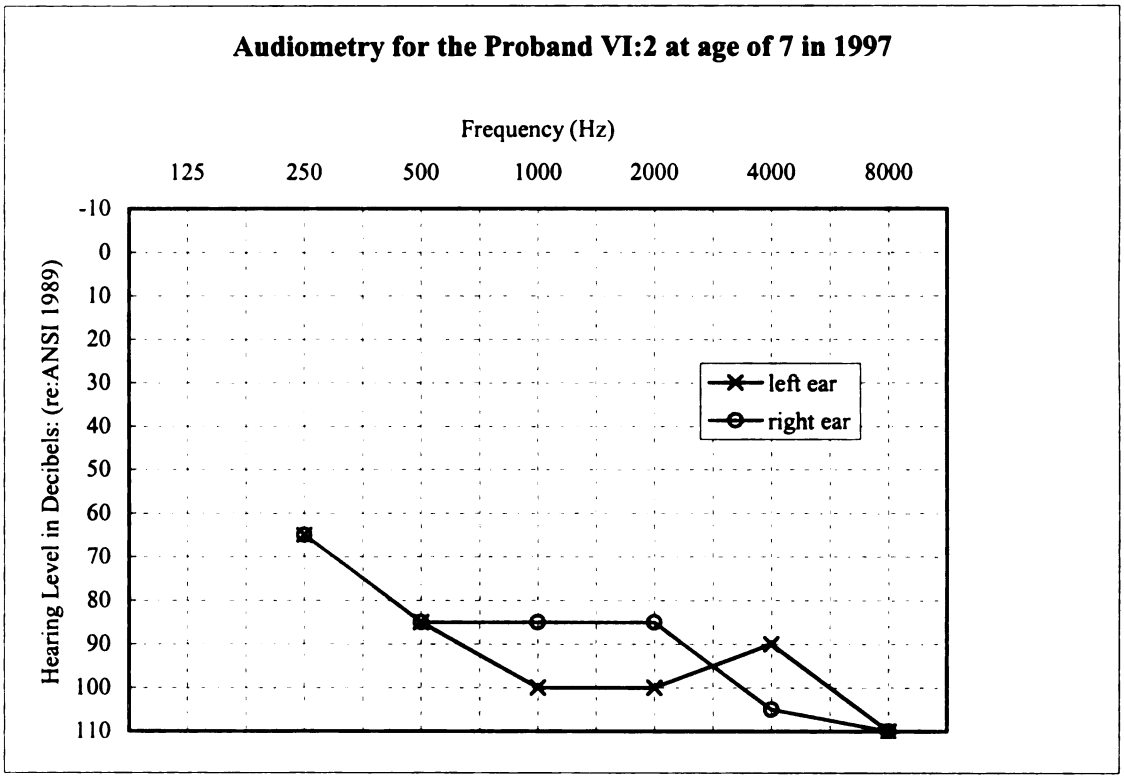


Figure 2. Pure-tone audiometry for the proband from the pedigree. Hearing loss (dB) is shown for both the right ear (O) and left ear (x), at a range of frequencies.



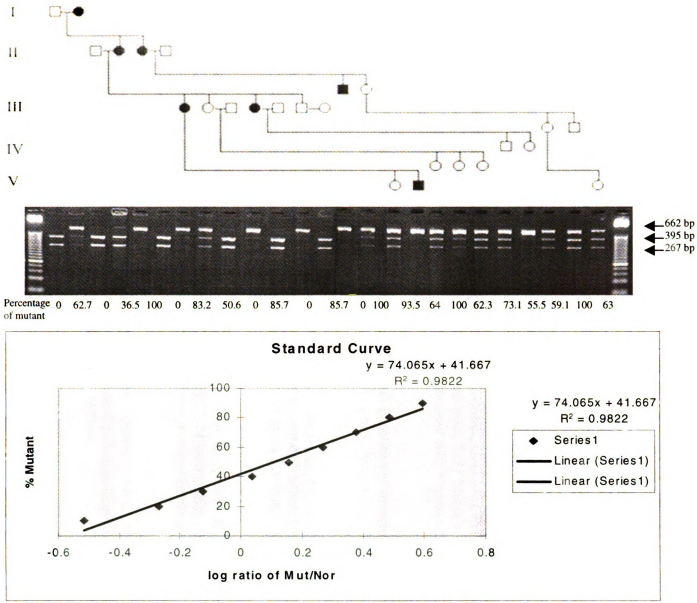


Figure 3. Standard curve and partial pedigree together with gel image and quantified mitochondrial

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