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IDENTIFICATION OF A GENETIC CONTRIBUTION TO MENIERE'S DISEASE

by

Colleen Ann Campbell

An Abstract

Of a thesis submitted in partial fulfillment
of the requirements for the Doctor of
Philosophy degree in Genetics
in the Graduate College of
The University of Iowa

May 2010

Thesis Supervisor: Professor Richard JH Smith

ABSTRACT

Ménière's disease (MD) is a complex disorder of the inner ear characterized by the symptoms of hearing loss, tinnitus, and vertigo, with an incidence in Caucasians of one in 1000. The hallmark histopathologic feature of MD is endolymphatic hydrops. Symptoms of MD typically present in the fourth decade of life, and the vertigo attacks experienced by patients with MD can be debilitating. Treatments aimed at alleviating the symptoms of MD are ineffective in approximately 30% of patients. Several studies have attempted to identify genetic factors important in MD through the use of families segregating the disease, but causative genes have not been identified. Many of these studies have been unsuccessful due to the fact that families of sufficient size to generate meaningful linkage results are extremely rare. Attempts to identify a genetic component to MD through the use of candidate gene association studies have been underpowered or poorly designed and therefore also unsuccessful.

We hypothesize Ménière's disease is a complex disorder that is due to the interplay of genetic and environmental factors. We tested this hypothesis using linkage and association studies. Initially, we focused on candidate gene replication association studies (*KCNE1*, *KCNE3*, *iNOS*), as well as testing a novel candidate gene (*AQP4*). We were unable to replicate the previous associations and although we could not identify an association between MD and *AQP4* we did discover rare variants of *AQP4* in our MD patient population. These variants segregate with a 'syndromic' MD phenotype.

We also performed a genome-wide linkage study on a large Chilean family segregating MD over three generations and identified a novel MD locus on 1q32.1-1q32.3. Targeted exon capture and pyrosequencing of the region identified two potential disease-causing variants in two genes of unknown function. We next screened a cohort of singleton patients with MD for variants in these same genes. Surprisingly, in both genes, we identified common and rare variants supporting a possible role for either gene

in the development of MD. The function of these two genes is unknown. Our results imply that additional studies must be undertaken to determine whether one or both genes has a role in the pathogenesis of MD. Identification of a causative gene will aid in the understanding of disease pathophysiology and lead to improved treatments.

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Graduate College
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CERTIFICATE OF APPROVAL

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PH.D. THESIS

____

This is to certify that the Ph.D. thesis of

Colleen Ann Campbell

has been approved by the Examining Committee
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To my parents

Do not go where the path may lead, go instead where there is no path and leave a trail.

Ralph Waldo Emerson

1803-1882

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ABSTRACT

Ménière's disease (MD) is a complex disorder of the inner ear characterized by the symptoms of hearing loss, tinnitus, and vertigo, with an incidence in Caucasians of one in 1000. The hallmark histopathologic feature of MD is endolymphatic hydrops. Symptoms of MD typically present in the fourth decade of life, and the vertigo attacks experienced by patients with MD can be debilitating. Treatments aimed at alleviating the symptoms of MD are ineffective in approximately 30% of patients. Several studies have attempted to identify genetic factors important in MD through the use of families segregating the disease, but causative genes have not been identified. Many of these studies have been unsuccessful due to the fact that families of sufficient size to generate meaningful linkage results are extremely rare. Attempts to identify a genetic component to MD through the use of candidate gene association studies have been underpowered or poorly designed and therefore also unsuccessful.

We hypothesize Ménière's disease is a complex disorder that is due to the interplay of genetic and environmental factors. We tested this hypothesis using linkage and association studies. Initially, we focused on candidate gene replication association studies (*KCNE1*, *KCNE3*, *iNOS*), as well as testing a novel candidate gene (*AQP4*). We were unable to replicate the previous associations and although we could not identify an association between MD and *AQP4* we did discover rare variants of *AQP4* in our MD patient population. These variants segregate with a 'syndromic' MD phenotype.

We also performed a genome-wide linkage study on a large Chilean family segregating MD over three generations and identified a novel MD locus on 1q32.1-1q32.3. Targeted exon capture and pyrosequencing of the region identified two potential disease-causing variants in two genes of unknown function. We next screened a cohort of singleton patients with MD for variants in these same genes. Surprisingly, in both genes, we identified common and rare variants supporting a possible role for either gene

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LIST OF ABBREVIATIONS

95%; CI= 95% confidence interval

A= Association study

A.A.= Amino Acid

AAO-HNS = American Academy of Otolaryngology-Head and Neck Surgery

ADD= Adducin

ALDH7A1= Aldehyde Dehydrogenase 7 Family, Member A1

AllDiffs= All Variants (Differences)

AQP= Aquaporin

ATQ1= Antiquitin

BAC= Bacterial artificial chromosome

BMD= Bilateral Ménière's disease

bp= Base Pairs

C=Candidate gene screen

CAPZA= F-actin-capping protein subunit

CDCV= Common disease Common Variant hypothesis

CDK18= Cell division protein kinase 18

cDNA= complementary DNA

CDRV= Common disease Rare Variant hypothesis

cM= centiMorgan

CNDI= Congenital Nephrogenic Diabetes Insipidus

COCH= Coagulation factor C Homology

CR1= Complement Receptor 1

CTLA4= Cytotoxic T-lymphocyte-associated protein 4

dChip= DNA-Chip Analyzer software

DFNA= Autosomal dominant nonsyndromic sensorineural hearing loss

DNA= Deoxyribonucleic acid

EH= Endolymphatic Hydrops

ES cell= Embryonic Stem Cell

EST= Expressed Sequence Tag

FLJ22655= Homo sapiens cDNA: FLJ22655 fis

FMD= Familial Ménière's disease

GAPDH= Glyceraldehyde-3-phosphate dehydrogenase

GCOS = GeneChip Operating Software

GDAS= GeneChip DNA Analysis Software

gDNA= Genomic DNA

GWA= Genome-Wide Association

GWAS= Genome-Wide Association Study

HapMap CEPH= Utah residents with ancestry from northern and western Europe

HapMap JPT= HapMap Japanese in Tokyo

HCDiffs= High Confidence Differences

HCFC1= Host cell factor C1

HL= Hearing Loss

HLA= Human Leukocyte Antigens

HSP= Heat Shock Protein

HSV-1 = Herpes simplex type one

HWE= Hardy-Weinberg Equilibrium

IBS= Irritable Bowel Syndrome

IHC= Immunohistochemistry

IHS= International Headache Society

iNOS= inducible Nitric Oxide Synthase

ISH= in situ hybridization

kb= Kilobase

KCNA1= Potassium channel, voltage-gated, Shaker-related subfamily, member 1

KCNE= Potassium channel, voltage-gated, ISK-related subfamily

KCNQ1= Potassium voltage-gated channel, KQT-like subfamily, member 1

L= Linkage study

LCT= Lactase

LD= Linkage Disequilibrium

LFSNHL= Low-Frequency Sensorineural Hearing Loss

LOC642587= Hypothetical protein LOC642587

LOD= Logarithm of the odds

LPS= Lipopolysaccharide

MAD= Migraine-associated dizziness, migrainous vertigo

Mb= Megabase

MD= Ménière's disease

Met= Methionine

MHC= Major Histocompatibility Complex

MMD= Migraine + Ménière's disease

Mt=mitochondrial

N.A.= Not Applicable

NDI= Nephrogenic Diabetes Insipidus

NF-KB= Nuclear Factor Kappa-light-chain-enhancer of activated B cells

NI= Non-Informative

OR= Odds Ratio

P6= Postnatal day 6

PARP-1=Poly (ADP-ribose)-polymerase 1

PCR= Polymerase Chain Reaction

PCTK3= PCTAIRE 3

PIK3C2G= Phosphatidylinositol 3-kinase, class 2, gamma

PLCZ1= Phospholipase C, zeta-1

PSMD4= Proteasome 26S subunit, non-ATPase, 4

PTPN22= Protein Tyrosine Phosphatase, Nonreceptor-type, 22

qPCR= Quantitative PCR

R=Replication association study

RERGL= Ras-related and estrogen-regulated growth inhibitor-like protein

RNA= Ribonucleic acid

RR= Relative Risk

RT-PCR= Reverse-Transcriptase-PCR

SLC26A4= Solute carrier family 26, member 4

SLC45A3= Solute carrier family 45, member 3

SNHL= Sensorineural Hearing Loss

SNP= Single Nucleotide Polymorphism

SNV= Single Nucleotide Variant

SPAB= Spontaneous Abortion

STRP= Short Tandem Repeat Polymorphism

T= Tinnitus

Thr= Threonine

Trp= Tryptophan

UK= United Kingdom

UTR= Un-translated Region

V= Vertigo

VP1= Enteroviral Antigen

WNL= Within Normal Limits

χ^2= Chi-square tests of independence

CHAPTER I

INTRODUCTION

Thesis Hypothesis & Goals

We hypothesize Ménière's disease is a complex disorder that is due to the interplay of genetic and environmental factors. We will test this hypothesis through the use of multiple genetic methods such as linkage and association studies.

In the short term, we expect the results of this study to identify the major genes that play a role in the etiology of MD. Our long term goal is to develop novel and more effective treatments for MD that are based on the genetics and pathophysiology of this disease.

Abstract

Ménière's disease (MD) is a complex idiopathic disorder of the inner ear characterized by the symptom triad of vertigo, sensorineural hearing loss and tinnitus. As defined by the American Academy of Otolaryngology-Head and Neck Surgery (AAO-HNS), the diagnosis is exclusionary and requires the documentation of two or more attacks of vertigo lasting more than 20 minutes, hearing loss and tinnitus or aural fullness. The histopathologic feature is endolymphatic hydrops.

Most cases of MD are sporadic although in a few families the disease segregates in an autosomal dominant fashion. Linkage studies to identify genetic factors important in disease pathogenesis have been unsuccessful, perhaps reflecting the complexity of MD. In contrast, several small association studies have identified potential genetic contributions to MD but larger cohorts must be analyzed to validate these results before any conclusions regarding the role of these genes in the pathogenesis of MD can be made. The purpose of this chapter is to review the biology and genetics of Ménière's disease with a focus on past and future genetic studies. This chapter has been submitted as an invited review to Hearing Research (Campbell and Smith In Press).

Overview - Description & Diagnosis

Prosper Ménière described the disease that carries his name in 1861 (Meniere 1861). A complex idiopathic disorder of the inner ear, Ménière's disease (MD) is a characterized by the symptom triad of vertigo, sensorineural hearing loss and tinnitus. As defined by the American Academy of Otolaryngology-Head and Neck Surgery (AAO-HNS), the diagnosis of 'definite' MD is exclusionary and requires the documentation of two or more attacks of spontaneous vertigo lasting more than 20 minutes, hearing loss documented on at least one occasion, and tinnitus or aural fullness in the affected ear. 'Certain' MD can only be diagnosed on autopsy and includes the above criteria plus histopathologic confirmation of endolymphatic hydrops (EH). The hallmark histopathologic feature of MD is endolymphatic hydrops. It is unknown if endolymphatic hydrops is due to abnormal production or absorption of endolymph (Mancini, Catalani et al. 2002). Endolymphatic hydrops can have multiple causes, and is classified as idiopathic when there is no obvious temporal bone abnormality and other causes have been excluded based on physical exam, clinical history, serologic testing for syphilis, and ophthalmologic consultation (1995).

'Probable' MD should be considered when one attack of vertigo has occurred with documented hearing loss and tinnitus or aural fullness in the affected ear. 'Possible' MD is considered if MD-type vertigo occurs without hearing loss or if fluctuating sensorineural hearing loss without vertigo is documented (1995). Early in the disease course, hearing loss does not necessarily fluctuate, but over time, hearing deteriorates and concomitantly, vertiginous episodes become less severe and often cease completely (Silverstein, Smouha et al. 1989; Stahle, Friberg et al. 1991; 1995; Morrison 1995). 30-50% of MD patients develop bilateral symptoms within 2 to 20 years of presenting with unilateral symptoms (Wladislavosky-Waserman, Facer et al. 1984; Sajjadi 2002), although some patients may experience bilateral disease from the onset of symptoms (Stahle, Friberg et al. 1991).

Symptoms typically present in the 4th decade, affecting both genders equally (Morrison 1995). The inciting event of MD is unknown, and following onset of disease, patients may experience symptoms for days or months, or be symptom-free for years (Paparella and Djalilian 2002). Vertigo can severely impact many activities of daily living (Mancini, Catalani et al. 2002). Because there is no standard diagnostic test for MD, it is over-diagnosed by non-specialists (Thirlwall and Kundu 2006) and even amongst specialists the diagnosis can be difficult to make (Thorp, Shehab et al. 2003; Kim, Wiet et al. 2005).

All treatments for MD are directed at symptomatology and include dietary restrictions, steroids, diuretic therapy and vestibular rehabilitation exercises. About 70% of patients benefit from this approach, but patients with intractable vertigo may require vestibular nerve sectioning or labyrinthectomy for relief (Saeed 1998; Sajjadi 2002; Thorp, Shehab et al. 2003; Kim, Wiet et al. 2005; Thirlwall and Kundu 2006).

Environmental Triggers

Both environmental and genetic factors are probably required for the development of MD (Morrison, Mowbray et al. 1994). Reported 'triggers' include emotional stress, anxiety and sudden head movement (Morrison 1995; Salim, Becker et al. 2007). A psychological assessment of 110 definite MD patients identified no personality abnormalities, although half had another chronic disease (van Cruijsen, Jaspers et al. 2006). Derebery and Berliner found a higher prevalence of environmental allergies in MD patients as compared to the general population (Derebery and Berliner 2000).

Morrison and colleagues studies viral triggers and detected an enteroviral antigen, VP1, more frequently in MD patients than controls during times of active disease (Morrison, Mowbray et al. 1994). Herpes simplex type one (HSV-1) viral DNA has also been identified in two of five MD patients (Morrison, Mowbray et al. 1994). Other reported 'triggers' include fungal middle ear infections (McMillan 2005), head trauma,

(Morrison, Mowbray et al. 1994), high salt diet, and the weather (cold-fronts) (Mizukoshi, Watanabe et al. 1995) and autoimmunity (Alleman, Dornhoffer et al. 1997).

Genetic Evidence

MD is a complex disease in which both environmental, and genetic factors may be necessary for development of symptoms (Brown 1949; Oliveira and Braga 1992; Morrison 1995). We believe a genetic predisposition is necessary for the development of MD, due to the following evidence; familial clustering, twins, and differences in incidence between populations.

Familial Clustering

Sporadic and familial MD are clinically indistinguishable and although most cases of MD are sporadic, in a few families a MD-like phenotype segregates in multiple persons in an autosomal dominant manner (Oliveira and Braga 1992). In fact, it is estimated that 5-14% of MD patients have an affected relative (Mizukoshi, Ino et al. 1979; Morrison 1981; Martini 1982; Birgerson, Gustavson et al. 1987; Morrison 1995; Morrison and Johnson 2002). Brown first recognized this familial component to MD and reported it in 1941; eight years later she described additional MD families including a pair of identical twins with the disease (Brown 1949; Oliveira and Braga 1992). Brown and Bernstein's early reports of familial MD include the added observation of migraine in several of these families (Brown 1941; Bernstein 1965). Inheritance is typically autosomal dominant, although in some small families autosomal recessive or maternal inheritance cannot be ruled out (Brown 1941; Birgerson, Gustavson et al. 1987; Oliveira and Braga 1992; Fung, Xie et al. 2002; Frykholm, Larsen et al. 2006; Klockars and Kentala 2007; Morrison, Bailey et al. 2008).

Penetrance is estimated to be 60-90% and expressivity can be variable, with vestibular dysfunction being the most commonly reported symptom of the symptom triad possibly due to environmental factors or modifier genes (Morrison 1995; Frykholm,

Larsen et al. 2006). Of persons with 'partial' disease, some progress to complete MD (Morrison 1995). Klockars and colleagues found in the Finnish population approximately 15% of individuals diagnosed with definite MD (AAO-HNS 1995 criteria) had a family history of relatives with definite MD or had relatives with symptoms consistent with partial disease, but had not yet been diagnosed with MD. Those with a family history tended to have more severe symptoms than those without a family history. In Finland, a quarter of the eight familial MD pedigrees analyzed independently segregated otosclerosis (Klockars and Kentala 2007).

In 10% of families, anticipation is described in which successive generations may have an earlier onset of symptoms and more severe disease although it has not been confirmed and may reflect ascertainment bias (Morrison, Mowbray et al. 1994; Morrison 1995; Fung, Xie et al. 2002; Frykholm, Larsen et al. 2006; Morrison, Bailey et al. 2008). Although MD is rare in children, individuals diagnosed with MD under age 20 are more common in families segregating the disease than in sporadic disease, (7.8% versus 2.7% respectively), and individuals have a greater likelihood of developing bilateral disease (Morrison 1995). If siblings are affected with MD the age of onset is similar with a mean difference of 6.16 years (Morrison 1995; Choung, Park et al. 2006).

Twins

Although there have been reports of twins with MD concordance rates in monozygotic versus dizygotic twins have not yet been reported, nor has heritability (Bernstein 1965; Comacchio, Boggian et al. 1992; Morrison 1995). Comacchio and colleagues reported twin brothers who inherited congenital nephrogenic diabetes insipidus (CNDI) and MD independently. Three additional sets of twins (2 monozygotic – determined by microsatellite markers; and one dizygotic) from 3 different families were identified in which one twin had MD and migraine and the other had episodic vertigo and migraine but no auditory symptoms (Cha, Kane et al. 2008).

Prevalence and Incidence

Although estimates of MD are difficult to obtain because its natural history and clinical variability impact diagnostic accuracy (Morrison 1995), prevalence and incidence clearly vary across the world and are highest in persons of Northern European descent (Table 1) (Morrison 1995). In the United States, incidence is estimated at 15-46 per 100,000 yearly, with a prevalence of 218 per 100,000 (Wladislavosky-Waserman, Facer et al. 1984; Wittner 2006). The incidence in Japan is lower (3.5 and 16 per 100,000), and in West Indian and native Americans, MD is rare (Wiet 1979; Wladislavosky-Waserman, Facer et al. 1984; Morrison 1995). MD is also rare in Uganda (Nsamba 1972), although in three other African countries the incidence may be higher: in Nigeria, Okafor and colleagues reported the incidence of MD to be 0.4% using modified diagnostic criteria (Okafor 1984); in Ghana, 6.1% of patients with hearing loss were considered MD patients, although the diagnostic criteria were not described (Amedofu, Ocansey et al. 2006); and in West Africa, the prevalence of MD was reported to be 0.22% although again diagnostic accuracy was questionable (Ibekwe and Ijaduola 2007). In Brazil, the incidence of MD is lower than it is in Europe and North America, most likely reflecting the ethnic diversity of the Brazilian population (Oliveira, Ferrari et al. 2002).

Table 1. Worldwide Incidence and Prevalence of Ménière's Disease. The incidence and prevalence results reported in this review are those reported by the original authors.

Population	Incidence	Prevalence	Reference
Finland	4.3 / 100,000	43.2 / 100,000	(Kotimaki, Sorri et al. 1999)
Finland (Southern)		513 / 100,000	(Havia, Kentala et al. 2005)
Great Britain	157 / 100,000 (0.16%)		(Cawthorne and Hewlett 1954)
Great Britain	1 / 1000		(Harrison 1968; Morrison 1995)
Ireland	10-20 / 100,000		(Wilmot 1979)
Italy (Southeastern)	8.2 / 100,000	205 / 100,000	(Celestino and Ralli 1991)
Japan (Toyama Prefecture)		17 / 100,000	(Watanabe, Mizukoshi et al. 1995)
Japan (Hida district)		36.6 / 100,000	(Shojaku and Watanabe 1997)
Japan (Nishikubiki district)		21.4 / 100,000	(Shojaku and Watanabe 1997)
Japan	3.5 - 16 / 100,000		(Morrison 1995)
Southwestern American Indians	Rare		(Wiet 1979)
Sweden	46 / 100,000		(Stahle, Stahle et al. 1978)
Uganda	Rare		(Nsamba 1972)
United States (Caucasian)	15.3 / 100,000	218.2 / 100,000	(Wladislavosky-Waserman, Facer et al. 1984)
United States (Framingham Heart Study)		1.48 %	(Moscicki, Elkins et al. 1985)
West African sub-region	0.0022		(Ibekwe and Ijaduola 2007)
West India	Rare (1 Jamaican of mixed blood in >2000 pts)		(Morrison 1995)

Overview of Ménière's Disease: Genetic Studies

Linkage Studies

A linkage study results in a genetic relationship between loci, whereas a genetic association is a statistical observation between alleles or phenotypes. Due to the numerous causes of adult onset hearing loss, tinnitus, and vertigo, recalling a family history of MD can be misleading, and there are very few large families that segregate the disease and are informative enough for a genetic study (Morrison, Mowbray et al. 1994). Due to the scarcity of large families segregating MD, very few linkage studies have been performed. Please see Appendix A for a more detailed discussion of linkage and association studies.

COCH

Fransen and colleagues completed one such study on a large Belgian family segregating progressive sensorineural hearing loss (SNHL) with progressive vestibular dysfunction. A P51S missense mutation in *COCH* was identified in affected family members and since more than one-fourth also had symptoms of vertigo, aural fullness, and/or tinnitus, the authors initially concluded that these patients fulfilled AAO-HNS 1995 diagnostic criteria for MD. They then recommended *COCH* mutation analysis in patients with sporadic MD. However, the authors noted there are subtle clinical differences between MD and DFNA9 patients (Fransen, Verstreken et al. 1999).

DFNA9 (*COCH*) and MD differ phenotypically as the former usually present with bilateral, progressive high frequency hearing loss, while the latter usually present with unilateral, fluctuating low frequency hearing loss (Usami, Takahashi et al. 2003). Usami and colleagues screened 20 Japanese patients with sporadic MD (AAO-HNS 1995 criteria) for mutations in *COCH* and failed to find any mutations in this cohort leading the authors to conclude mutations in *COCH* are not a major cause of sporadic MD

(Usami, Takahashi et al. 2003). Sanchez and colleagues confirmed Usami's findings in an analysis of another 30 MD patients (only COCH exons 4 and 5 were analyzed) (Sanchez, Lopez-Escamez et al. 2004).

12p12.3

Another linkage study was completed by Klar and colleagues using three unrelated Swedish families (AAO-HNS 1995 diagnostic criteria were utilized). A genome wide linkage scan performed on the first family with microsatellite markers identified five loci with a lod score >1. The authors excluded the following loci for linkage; *ATQ*, *HLA*, *SLC26A4*, *PSMD4*, *COCH*, *AQP1-12*, and two LFSNHL loci on chromosomes 1 and 4. A peak lod score of 2.43 on 12q15 was identified in the first family, and a cumulative lod score of 2.76 at 12p12 was found with the additional two families, with families 1 and 2 sharing a common haplotype over seven markers in a 7Mb region. Using family 3 the linked region was narrowed first to a 725kb interval that includes *FLJ22655 (RERGL)*, *PIK3C2G*, *PLCZ1*, and *CAPZA*, and then with additional markers to a 463kb interval containing *RERGL* and *PIK3C2G*. To date, the MD gene in this interval has not been reported, and although a mutation in *PIK3C2G* was not identified the authors conclude further investigation into mutations of regulatory regions and deletions needs to be performed (Klar, Frykholm et al. 2006). Frykholm and colleagues describe what appears to be the same Swedish family as family 1 described by Klar. In this study microsatellite markers were used to rule out linkage to DFNA1, DFNA6/14, DFNA9, and DFNA15 (Frykholm, Larsen et al. 2006).

Association Studies

Association studies compare frequencies of specific alleles between a test population and a control population using either a candidate gene or a genome-wide association study design. As a general rule, an association study is a powerful method to identify genetic components of a complex disease. If a candidate gene study design is

selected it presumes picking an appropriate candidate based on putative function, expression, and role in disease pathophysiology. A genome-wide association study (GWAS), in contrast, is not hypothesis driven (Cardon and Bell 2001). The results of an association study can indicate an allele is in linkage disequilibrium with the disease causing allele, causes biologic susceptibility to disease, or be a false positive result (Morrison and Johnson 2002). Association study statistics are highly influenced by sample size and studies of small cohorts should be interpreted cautiously. Presented in chronological order are the results of recent association studies for MD.

HLA

Xenellis and colleagues have reported an association between MD and HLA-CW7 (Human Leukocyte Antigens) in a British MD population. (41 classical MD patients; 187 unrelated Caucasian controls) After correcting for multiple antigen testing, HLA-CW7 differed significantly between patients and controls suggesting an autoimmune component to MD (p=0.035). Factors such as gender, laterality of disease (uni or bilateral), and left- or right-sided disease did not result in a difference in antigen frequency. The authors also suggested an alteration of the complement system may cause MD. Finally, the authors warned although the HLA-CW7 allele may cause MD, the association may be a reflection of linkage disequilibrium with the disease-causing gene (Xenellis, Morrison et al. 1986). Morrison found HLA-A3, Cw7, B7, and DR2 were more frequent in familial MD (FMD) patients than the general population (Morrison, Mowbray et al. 1994). Individuals with FMD also had a higher frequency of HLA-A2 and HLA-B44 antigens than those without a family history (Arweiler, Jahnke et al. 1995).

Fung and colleagues performed linkage analysis on six individuals from two families segregating MD with markers to the HLA region on chromosome 6, but did not find a shared haplotype between the affected individuals (Fung, Xie et al. 2002). Yeo and colleagues completed HLA-A, -B, -C typing in a Korean population (39 MD patients

and 199 healthy Korean controls – it was not stated whether the controls were matched by gender and age to the patients) (Yeo, Park et al. 2002). None of the associated alleles from previous studies, except, HLA-DRB1*15, differed between cases and controls in this study. HLA-Cw*0303, HLA-Cw*0602, and HLA-DRB1*15 were more frequent in MD patients than controls (RR=2.5, $p<0.02$; RR=3.7, $p<0.03$, RR=2.4, $p<0.03$, respectively). While it is important to note that no corrections were made for multiple allele testing, these results suggest that HLA allele associations may be population specific (Yeo, Park et al. 2002).

Koo and colleagues sought to determine if HLA-DR alleles were associated with MD if type II collagen antibody status was taken into consideration. The authors studied 41 Korean patients diagnosed with definite or probable MD by AAO-HNS 1995 criteria and 226 Korean controls matched for gender and age. All HLA-DRB1 association tests were corrected for multiple comparisons (i.e. Bonferroni correction). MD patients had a significantly higher level of anti-CII antibody level and sero-positive rate as compared to controls ($p=0.005$ and $p=0.002$, respectively). Initial analysis of HLA-DRB1*0405 showed an association (increased frequency) with anti-CII positive patients compared to controls and to anti-CII negative patients, but only a small number of MD patients were anti-CII positive (n=8, 20%) and the association did not withstand the Bonferroni correction. Several additional alleles showed an initial association, but after Bonferroni correction none of the HLA-DR alleles was associated with MD (Koo, Oh et al. 2003).

In aggregate, these data suggest that if the HLA region leads to a genetic predisposition for MD, it is unlikely that there is a single HLA allele associated with MD across different populations (Morrison, Mowbray et al. 1994; Fung, Xie et al. 2002; Paparella and Djalilian 2002). It is also possible that the inconsistent results are spurious, although it is difficult to make a broad conclusion as the study designs vary.

<u>KCNE1 & KCNE3</u>

Doi and colleagues chose *KCNE1* and *KCNE3* for a candidate gene association study based on their cellular expression and role in ion and water transport (Doi, Sato et al. 2005). *KCNE1* is expressed in the marginal cells of the stria vascularis, and *KCNE3* is expressed in the distal portion of the endolymphatic sac in the epithelium. Sequence analysis of the coding regions of both genes was completed in 63 definite MD patients (AAO-HNS 1995) and 237 (*KCNE1*) and 205 (*KCNE3*) controls matched for age and gender. Statistical analysis included chi-square test and Fisher's exact test with $p<0.05$ considered significant (Doi, Sato et al. 2005). In *KCNE1*, the A allele of rs1805127 was found more frequently in patients than controls ($p<0.001$) while in *KCNE3*, the C allele of rs2270676 was found more frequently in patients than in controls ($p=0.0015$), leading the authors to conclude that variation in *KCNE1* and/or *KCNE3* increase susceptibility for sporadic MD (Doi, Sato et al. 2005).

Campbell and colleagues attempted to replicate these associations in a Caucasian MD population from the United States (Campbell, Della Santina et al. 2010). Sanger sequencing for the coding regions of both genes was completed on DNA from 180 singleton Caucasian, non-Hispanic definite MD patients (AAO-HNS 1995 criteria) and 180 controls matched for gender, age, race, and ethnicity. Population stratification within the patient and control cohorts was excluded. The authors also screened a Japanese control group obtained from Okayama University (Okayama CTRLS) (*n*=134, *KCNE1*; *n*=131, *KCNE3*), and used allele and genotype frequencies reported for the HapMap Japanese in Tokyo (HapMap JPT) and Utah residents with ancestry from northern and western Europe (HapMap CEPH). Chi-square tests of independence with a Yate's correction for continuity if necessary were used with p-values ≤0.05 considered significant for all novel and reported variants.

Neither polymorphism was associated with MD in the Caucasian population (*KCNE1*, p= 0.55; *KCNE3*, p= 0.870). In addition, none of the other variants or

haplotypes screened in either gene were associated with MD in Caucasians. The inability to replicate these associations was not due to allele differences between Caucasian and Japanese MD patients (*KCNE1*, p=0.903; *KCNE3*, p=0.862). Comparison between the Japanese MD patients and the second Japanese control cohort (Okayama controls) revealed less robust associations (*KCNE1*, p=0.024; *KCNE3* p= 0.025), and no association when compared to the HapMap JPT population (*KCNE1*, p=0.781; *KCNE3*, p=0.252). The controls used in the Doi et al study deviated from Hardy-Weinberg equilibrium (HWE) (*KCNE1*, $p=1.33x10^{-5}$; *KCNE3*, p=0.035) indicating population admixture or genotyping errors. The authors conclude the reason for the inability to replicate the reported associations is most likely due to an inappropriate control cohort for the patients in the study by Doi and colleagues. Overall, the authors conclude replication of reported associations is important in determining which candidate genes and polymorphisms to pursue for functional studies and genetic testing. In addition, variations in *KCNE1* and *KCNE3* are not associated with MD in Caucasians (Campbell, Della Santina et al. 2010).

HLA-DRB1*1101

Lopez-Escamez and colleagues performed a prospective multicenter case-control association study to determine if HLA-DRB1* (6p21.32) and HLA-DQB1* (6p21.32) Class II alleles of the major histocompatibility complex are associated with bilateral MD (BMD) in two different ethnic groups from Spain. The authors proposed polymorphisms in these genes may lead to an altered immune response. The first cohort consisted of 37 definite bilateral MD patients from the Galicia area in northwest Spain (AAO-HNS 1995 criteria); and the second cohort consisted of 43 definite bilateral MD patients from southeast and eastern (Mediterranean) Spain. The patients represent two distinct ethnic groups and were therefore analyzed separately. DNA from 145 controls from Galician and 105 controls from southeast Spain (Mediterranean) was analyzed. Allele-specific

amplification and sequence-based HLA typing were performed on all participants, antigen frequencies determined, and Fisher's exact test with corrections for multiple testing utilized. The HLA-DRB1*1101 allele was found in 19% of the Mediterranean patients (n= 15) and 6% of the Mediterranean controls (n=11), resulting in an odds ratio of 3.65 (95% confidence interval, 1.5-9.1), corrected p=0.029. The HLA-DRB1*11 allelic group was also associated with this cohort, OR=3.30 (95% confidence interval, 1.5-7.8), corrected p=0.012. No associations were seen for the Galicia cohort for HLA-DRB1* alleles, and no associations were seen for either cohort with HLA-DQB1* alleles. Overall, the sample size is small for each cohort with not many alleles representative for any given allele although the authors conclude that MHC Class II genes as a whole contribute to the development of bilateral MD and that persons from southern Europe with the HLA-DRB*1101 allele or HLA-DRB1*11 allelic group might be at higher risk for developing bilateral MD (Lopez-Escamez, Vilchez et al. 2007). The results of this study have not been replicated.

Host cell factor C1

Vrabec and colleagues used 69 single nucleotide polymorphisms (SNPs) to interrogate 39 candidate genes selected based on proposed pathogenic mechanisms of MD (viral infection or reactivation genes, familial migraine genes, familial cochleovestibular dysfunction, potassium transport, mediators of inflammation genes) (Vrabec, Liu et al. 2008). Twenty-one definite MD patients and 33 controls were used for the original genotyping; 30 patients and 40 controls were used for the second phase genotyping (AAO-HNS 1995 criteria). Non-polymorphic SNPs and SNPs not in Hardy-Weinberg equilibrium (HWE) were excluded from the study. They found that haplotypes of SNPs in *HCFC1* were significant, although the haplotypes and haplotype association data were not shown.

Because rare alleles of these SNPs were more frequent in controls than cases (rs762653: OR, 0.33 95%; CI, 0.13-0.81, rs17421: OR, 0.21; 95% CI, 0.07-0.65, rs2266886: OR, 0.26; 95% CI, 0.10-0.65), a protective effect was proposed, and Sanger sequencing of the coding regions of *HCFC1* was completed in 10 cases and 10 controls. This identified 36 SNPs, 17 of which were coding, including one non-synonymous variant, S1164P (rs1051152), and a novel variant hCV25623002 (IVS4-13). No associations were reported in the other candidate genes, and the study was unable to replicate the association identified by Doi and colleagues between rs1805127 in *KCNE1* and MD. The authors conclude *HCFC1* may be involved in the pathogenesis of MD as multiple SNPs in the gene were associated with MD. These results must be replicated in additional cohorts and complemented with functional studies before *HCFC1* can be implicated in MD (Vrabec, Liu et al. 2008).

α-Adducin

Adducin mutations are associated with hypertension. Teggi and colleagues proposed a common pathway between MD and hypertension may exist due to the common treatments of diuretics and low salt diet (Ferrandi, Salardi et al. 1999). Adducin is encoded by three genes (*ADD1, ADD2, ADD3*) each encoding a subunit of the protein (α, β, γ, respectively) to form a heterodimeric cytoskeleton protein. α-Adducin is encoded by *ADD1* on chromosome 4p16.3. Genotyping was completed on 28 Caucasian definite MD patients (AAO-HNS 1995 criteria) and two control groups; 48 normotensive age and gender matched controls; 1,713 Caucasian general population controls. In *ADD1*, G460W (rs4961) was found more frequently in MD patients than in controls (reported $\chi^2 = 4.46$, $p = 0.0034$ for matched controls; $\chi^2 = 5.29$, $p = 0.0013$ for general controls), leading the authors to conclude the 460Trp allele may be one of several predisposing risk factors for the development of MD (Teggi, Lanzani et al. 2008). The results of this study have not been replicated.

HSP70-1

Kawaguchi and colleagues genotyped two promoter SNPs in *HSP70-1* (HSPA1A) in 49 MD patients (AAO-HNS 1995 criteria) and 100 healthy controls. Increased levels of stress hormone have been reported in MD patients compared to controls and stress is a trigger of MD symptoms, therefore SNPs in HSP70 may alter an individual's sensitivity to stress thereby lending the individual more susceptible to disease. The C allele of 190 G/C was found more frequently in MD patients than in controls (χ^2 p-value <0.001). However, no difference was seen with this SNP and severity of disease suggesting MD is multifactorial and another factor determines disease severity. The authors conclude this association demonstrates that stress is indeed a trigger for MD and that HSPA1A 190C may be a cause for MD (Kawaguchi, Hagiwara et al. 2008). The results of this study have not been replicated.

PARP-1

Lopez-Escamez and colleagues genotyped a polymorphic repeat located in the promoter of *PARP-1 (*Poly (ADP-ribose)-polymerase 1) in 80 definite bilateral MD (BMD) patients (AAO-HNS, 1995) and 371 controls of the same origin. Chi-square tests with Yate's corrections, and Fisher's exact test were used with multiple testing corrections performed. Longer alleles, $(CA)_{17\text{-}20}$ were found less frequently in patients than controls (corrected p value = 0.012, OR=7.33, 95% CI, 1.77-30.37). The authors conclude the longer alleles of this polymorphism may protect against the development of BMD by allowing transcription factors to bind the promoter resulting in increased transcription of *PARP-1*. Since *PARP-1* is a transcriptional co-activator of NF-KB, this could lead to an increase in DNA repair and protection from cell death in the spiral ganglion neurons (Lopez-Escamez, Moreno et al. 2009). The results of this study have not been replicated.

PTPN22

PTPN22 encodes the protein lymphoid tyrosine phosphatase, also known as protein tyrosine phosphatase, nonreceptor-type, 22. The 1858 C/T variant (rs2476601) in *PTPN22* alters binding of this protein to Csk kinases, which may alter regulation of T cell activation and has been associated with other autoimmune diseases (Lopez-Escamez, Saenz-Lopez et al.). 52 Spanish bilateral MD patients (AAO-HNS 1995 criteria) and 348 controls were genotyped for rs2476601 (R620W). MD patients were more frequently heterozygous for this variant than controls (p-value 0.04, OR 2.25, CI 1.09-4.62), and the C allele (common allele) was more frequent in controls (p-value 0.05, OR 0.46, CI 0.22-0.94). The authors also genotyped rs231775 in *CTLA4* but the SNP was not associated with MD in their population. The authors conclude the association of rs2476601 in *PTPN22* lends support for BMD having an autoimmune component and also suggest the variant be studied in patients with unilateral disease (Lopez-Escamez, Saenz-Lopez et al.). The association has not been replicated.

Candidate Gene Studies

AQP2

Mhatre and colleagues characterized AQP2 expression in the cochlea and screened 12 MD patients for mutations in the coding regions of *AQP2* (12q13.13) to elucidate a role for AQP2 in MD. *AQP2* was chosen as a candidate gene due to its expression in the cochlea surrounding regions that border endolymph. In addition, *AQP2* is regulated by vasopressin which previously was demonstrated to be elevated in serum of patients with MD (Takeda, Kakigi et al. 1995). The authors found AQP2 expressed in Sprague-Dawley rat cochleae as well as adult CD1 mice cochleae, kidney, and testis. A cochlea specific transcript was not identified by RT-PCR or Western blot. The authors did not identify any mutations in the coding regions of *AQP2* or the intron-exon

boundaries. However, due to the small sample size the authors did not rule out *AQP2* as a candidate gene for MD (Mhatre, Jero et al. 2002).

ATQ1

Lynch and colleagues performed a candidate gene analysis of *ATQ1* (antiquitin) (5q23.2) due to its putative fluid regulatory function during stress and fetal inner ear and cochlear outer hair cell expression. Antiquitin is now referred to as Aldehyde Dehydrogenase 7 Family, Member A1 (*ALDH7A1*). Of the eight families studied, five were suggestive of autosomal dominant disease, and one segregated autosomal recessive disease. Standard AAO-HNS diagnostic criteria were not used (Lynch, Cameron et al. 2002). Screening of the exons and exon-intron boundaries for one patient from each family, a singleton MD patient and two controls revealed two polymorphisms in exon 14, but both were detected in 20 controls (IVS13-27 C allele 12.5% frequency, A1475C C allele 10% frequency). Southern blot analysis of a full length cDNA clone did not reveal any rearrangements of *ATQ1*, and enzyme digest of BAC DNA did not identify any trinucleotide repeats. The authors concluded the polymorphisms identified in this study were not disease causing, nor were genomic rearrangements of *ATQ1* (Lynch, Cameron et al. 2002).

Syndromic MD

First noted by Ménière and later by others, there is a higher prevalence of migraine and autoimmune disease in patients with MD than would be expected, both of which have associated genes lending support for a genetic predisposition to the development of MD (Brown 1941; Brown 1949; Morrison and Johnson 2002; Oliveira, Ferrari et al. 2002; Ruckenstein, Prasthoffer et al. 2002; Boyev 2005). Families segregating MD and migraine in an autosomal dominant manner have been reported and suggest a common genetic cause and a possible continuum between migraine and MD (Oliveira, Bezerra et al. 1997; Oliveira, Ferrari et al. 2002).

Ménière also noted similarities between glaucoma and MD. Nearly a century later, Godtfredsen and McGrath reported MD and glaucoma in the same patients and proposed a common origin for these two diseases (Godtfredsen 1949; McGrath 1952).

Expression Microarray

Sekine and colleagues used a custom gene expression microarray of stress related genes (n=1467) on DNA from two definite MD patients during a vertigo attack to assess stress response (AAO-HNS 1995 criteria) (Sekine, Morita et al. 2005). One patient had sporadic vertigo attacks, and the second patient had more severe disease with frequent vertigo attacks. Gene expression profiles during attack and active phases were compared to gene expression profiles during remission, and also compared to expression profiles of 5 control individuals following unilateral caloric stimulation. A list of genes investigated in this study on a website in Japanese.

Fifty-seven genes were found to be up-regulated >2 fold or down-regulated by less than half in the first patient, and 163 genes in the second patient. Twenty-six genes had altered expression in both patients during the attack and active phases but baseline expression during remission and not altered in controls. The authors state majority of these genes are inflammation or cytokine-related genes, and conclude these genes are involved with the development of vertigo attacks, but gene names are not provided. The authors conclude stress hormone products of the genes may lead to the development of vertigo attacks in patients with MD, and similar studies of larger cohorts of patients are necessary (Sekine, Morita et al. 2005).

Animal Models and MD

To date there is not a naturally occurring animal model of MD. The most common model involves surgical obliteration of the endolymphatic duct and endolymphatic sac with bone wax in guinea pigs to induce endolymphatic hydrops. Alternatively, endolymphatic hydrops can be induced immunologically by injection of

horseradish peroxidase antigen in the sac area, however this method does not consistently induce endolymphatic hydrops. Although both models induce EH, neither is a perfect model for MD as the animals do not demonstrate symptoms of vertigo (Lee, Ho et al. 1991). The Phex (Hyp-Duk)/Y hemizygous male mouse is a model for X-linked hypophosphatemic rickets and has spontaneous endolymphatic hydrops and hearing loss due to loss of spiral ganglion cells (Megerian, Semaan et al. 2008), however as MD in humans does not appear to be X-linked and the mouse does not have vertigo, so this is not an ideal model. Takumida and colleagues developed a model in which mice receive an intratympanic injection of lipopolysaccharide (LPS) to reduce absorption of endolymph, and intraperitoneal injection of aldosterone to induce an increase in endolymph production by stimulating Na/K ATPase in the stria vascularis. Injection of epinephrine into the middle ear is necessary to induce EH in this model. The authors conclude that vestibular dysfunction may not be the result of endolymphatic hydrops, alternatively, the induced animal models of endolymphatic hydrops may model the asymptomatic phase of MD, and additional factors such as stress may induce the symptom of vertigo experienced during an MD attack (Takumida, Akagi et al. 2007). In summary, there is not an animal model which exhibits all symptoms of MD as well as endolymphatic hydrops. When a pathogenic gene is identified for MD, an animal model with a mutant form of the gene may not exhibit the symptoms of MD without extrinsic factors such as stress or high salt diet. Therefore, full characterization of any future genetic animal models of MD must be carefully examined for phenotype at various ages as well as under various conditions before any conclusions can be drawn about the role of the gene(s) in the development of MD.

Summary

MD is a complex disease that appears to require genetic susceptibility as well as environmental triggers. The cause of MD remains unknown despite much research.

Although many good candidate genes have been chosen for association studies and candidate gene mutation studies, these studies have been small and have not yet been replicated. Therefore, reported associations should be interpreted cautiously. However, these studies do present candidate genes for further investigation. Replication of these studies is needed in larger, statistically significant, carefully defined cohorts.

A candidate gene association study requires the ability to select an appropriate candidate gene based on understanding of disease pathogenesis as well as appropriate study design features to detect an association. Results of association studies must be interpreted carefully. One should begin by analyzing the association study design as factors critical to design have a great impact to detect a true association and the ability to replicate an association in another cohort (Attia, Ioannidis et al. 2009).

The patient cohort should be carefully evaluated and the phenotype properly defined using AAO-HNS criteria. It is important to note that disease etiology may differ among ethnic groups. The control group must be matched to the cases preferably by gender, age, ethnicity, and ideally one control per patient. In addition, population stratification should be ruled out in both the case and control populations. Population stratification occurs if disease frequency varies with ethnicity, a marker found at a high frequency in one ethnic group may have a positive association with the disease phenotype even if it is not the causative allele or near a causative allele (Tsai, Choudhry et al. 2005). Finally, the controls should be in Hardy-Weinberg equilibrium (HWE), $p>0.05$. Genotyping errors or population admixture can result in deviations from HWE (Tiret and Cambien 1995; Salanti, Amountza et al. 2005).

Population size must be considered as small sample sizes can lead to spurious associations (Ioannidis, Ntzani et al. 2001). Since MD is an uncommon disease we recommend a sample size of at least 100 cases and 100 controls. Variants with a frequency of $<0.5\%$-0.05% are classified as rare and are detected by sequencing a candidate gene. If a gene contains 10 rare disease causing variants with a frequency of

0.001, sequencing a cohort of 100 has a 90% chance of identifying one variant (Li and Leal 2009). One must consider how the variant(s) were chosen for the study. Variants are either selected and genotyped, usually single nucleotide polymorphisms (SNPs) or the entire gene interrogated by sequence analysis in a test and control population (Tabor, Risch et al. 2002). Sometimes only a single or few SNPs are genotyped based on previous reports in the literature. Screening of the entire candidate gene is preferred as ethnic-specific variants may lead to an inability to replicate a previous association (Chanock, Manolio et al. 2007). This approach will also assist in evaluating if an associated SNP is in linkage disequilibrium with a disease-causing variant. Genotyping needs to be completed in >95% of the population. Corrections of multiple comparisons must be performed, and although conservative the Bonferroni method is commonly accepted. The association should be replicated in another population before any conclusions can be made with regards to the role of the gene in disease pathogenesis. Finally, the previous associations could be spurious (Lander and Schork 1994; Tabor, Risch et al. 2002). We have applied these criteria to the MD association studies presented in Table 2.

An alternative approach is a genome wide association study (GWAS) since very little is known about the pathophysiology of MD and an exhaustive candidate gene association would be difficult for any one group to complete. In designing a GWAS, it is important to carefully define case and control cohorts. Although a GWAS will detect common variants associated with MD, it is limited in that it is unable to detect rare variants which may have larger affects on a gene and lead to MD. Identification and careful characterization of families segregating MD large enough to generate a significant linkage signal will lead to the identification of additional candidate loci. With the advent of new sequencing technologies, deep re-sequencing of any candidate regions will be helpful to identify any rare pathogenic variants. Any variant or gene found to be associated with MD will need further analysis to determine how the gene impacts the

development to MD. The search and identification of genes that confer susceptibility to develop MD is worthwhile as the knowledge will help our understanding of the disease thereby leading to improved diagnostics and more effective treatments.

Table 2. Ménière’s Disease Genetics Investigations. The chromosomal location of genes analyzed in MD patients along with the type of study are listed below. Mt=mitochondrial; A=Association study; R=Replication association study; L=Linkage study; C=Candidate gene screen. Association study evaluation criteria: a=AAO-HNS diagnostic criteria; b=control group matched (^{1}gender, ^{2}age, ^{3}ethnicity); c=population stratification ruled out; d=controls in HWE; e= >100 patients; f= entire candidate gene screened; g=Bonferroni correction utilized; h=association replicated in another population; N.A.=not applicable.

Gene & Author/Chromosome	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	X	Y	Mt	Evaluation Criteria
COCH (Fransen, Verstreken et al. 1999)														L												N.A.
COCH (Usami, Takahashi et al. 2003)														C												N.A.
COCH (Sanchez, Lopez-Escamez et al. 2004)														C												N.A.
12p12.3 (Klar, Frykholm et al. 2006)												L														N.A.
HLA (Xenellis, Morrison et al. 1986)						A																				a; b^3
HLA (Fung, Xie et al. 2002)						L																				N.A.
HLA (Yeo, Park et al. 2002)						A																				a; b^3

Table 2-continued

HLA (Koo, Oh et al. 2003)						A																				a; $b^{1,2,3}$; g
KCNE1, KCNE3 (Doi, Sato et al. 2005)										A										A						a; $b^{1,2}$; f
HLA (Lopez-Escamez, Vilchez et al. 2007)						A																				a; b^{3}; g
HCFC1 (Vrabec, Liu et al. 2008)																							A			a; b^{3}; d; f
ADD1 (Teggi, Lanzani et al. 2008)		A		A						A																a; $b^{1,2,3}$
HSP70-1(Kawaguchi, Hagiwara et al. 2008)						A																				a
PARP-1 (Lopez-Escamez, Moreno et al. 2009)	A																									a; b^{3}; g
PTPN22, CTLA4 (Lopez-Escamez, Saenz-Lopez et al.)	A	A																								a; b^{3}
KCNE1, KCNE3 (Campbell, Della Santina et al. 2010)										R										R						a; $b^{1,2,3}$;c; d; e; f

Table 2-continued

AQP2 (Mhatre, Jero et al. 2002)											C														N.A.
ATQ1 (ALDH7A1) (Lynch, Cameron et al. 2002)				C																					N.A.

Patient accrual

All participants of this study were diagnosed at the University of Iowa and Johns Hopkins University with definite MD (AAO-HNS criteria) (1995). To date, 101 patients have enrolled in the study from the University of Iowa, and at least 218 patients from Johns Hopkins University. The patients are divided into matched test and replication cohorts. Currently, each patient cohort comprises 124 Caucasian, non-Hispanic singleton individuals. Upon enrollment the patients at the University of Iowa complete a basic medical history questionnaire relating to their MD symptoms (Appendix B). Of the patients from the University of Iowa, majority are white non Hispanic, 59 are female (58.4%), 42 are male (41.6%), mean age is 55.1 years with a mean age at diagnosis of 48.1 years. 78 patients have been diagnosed with definite MD, 2 with probable MD, and 2 with possible MD. Four patients report a family history of MD. Thirty-nine unrelated controls have also enrolled in the study at the University of Iowa (17 female, 22 male, mean age 56.1 years). In addition, we have a collection of DNA from ~100 Iranian patients with MD. To date this is the largest collection of DNA from MD patients we are aware of.

De-identified blood donor samples collected at the University of Iowa serve as controls for the study. Each patient is matched by gender and age with two controls. Patients over 70 years of age are matched with one control. Peripheral blood from all subjects was ascertained under Institutional Review Boards approved guidelines from the University of Iowa and Johns Hopkins University. Genomic DNA from all patients and controls was extracted by standard methods (Grimberg, Nawoschik et al. 1989).

CHAPTER II

TO EVALUATE CASE-CONTROL CANDIDATE GENE ASSOCIATION STUDIES FOR MÉNIÈRE'S DISEASE

Abstract

Candidate genes for association studies can be selected a variety of ways. Genes can be selected based on a hypothesis regarding disease pathogenesis, based on previous reports of genes or proteins being implicated with disease, or following a genome wide linkage or association study. In this chapter we have selected three genes based on previous reports (*KCNE1*, *KCNE3*, *iNOS*). In Chapter III we report the results from a gene selected based on expression in the literature and on a hypothesis regarding MD (*AQP4*). Due to our limited knowledge regarding the pathogenesis of MD it is extraordinarily difficult to select candidate genes as traditional filters such as protein function, gene and/or protein expression, or animals models are not possible to use.

Replication of *KCNE1* & *KCNE3* association with MD

The following part was recently published in the American Journal of Medical Genetics Part A (Campbell, Della Santina et al. 2010). The publication emphasizes the importance of study design for association studies and explanations why associations can fail to replicate in subsequent studies. There have been multiple associations reported for MD and although this study only attempts to replicate one of those reports, many of the reported associations may be spurious due to small sample sizes, inappropriate control groups, or population substructure in either the case or control cohort. The following manuscript also emphasizes the need for replication prior to initiating genetic testing or functional studies based on the results of an association study.

Abstract

Ménière disease (MD) is a complex disorder of unknown etiology characterized by the symptom triad of vertigo, sensorineural hearing loss and tinnitus. Its reported incidence is 1-2 per 1000 in Caucasians and 0.03-0.37 per 1000 in Japanese. Doi and colleagues recently reported that two single nucleotide polymorphisms (SNPs) in *KCNE1* and *KCNE3* are associated with MD in Japanese subjects (Doi, Sato et al. 2005). Consistent with this possibility, these two genes encode potassium channels that are expressed in the stria vascularis and endolymphatic sac, respectively, and their role in ion transport suggests that they may be important in inner ear homeostasis.

To establish whether a similar association exists in the Caucasian MD population, we sequenced the coding regions and exon-intron boundaries of both genes in 180 Caucasian persons with MD and 180 matched Caucasian controls. Neither of the two reported SNPs was significantly associated with MD when compared to the Caucasian controls (*KCNE1*, p= 0.55; *KCNE3*, p= 0.870). Comparison of allele frequencies between the Japanese MD population and our study population revealed no significant difference between groups (*KCNE1*, p=0.90; *KCNE3*, p=0.862), suggesting that the significant differences reported in the Japanese study arose from their control population. Six additional SNPs in both *KCNE1* and *KCNE3* were genotyped and none was associated with MD. Population stratification within our MD and Caucasian control population was excluded. Our data show that SNPs in *KCNE1* and *KCNE3* are not associated with MD in Caucasians.

Introduction

Ménière disease (MD) is a complex idiopathic disorder of the inner ear characterized by the symptom triad of vertigo, sensorineural hearing loss and tinnitus. It was first recognized by Prosper Ménière in 1861 and is now defined by the American Academy of Otolaryngology – Head and Neck Surgery as an exclusionary diagnosis that

requires the documentation of two or more attacks of vertigo lasting more than 20 minutes, hearing loss, and tinnitus or aural fullness (1995). However, the diagnosis can be difficult to make with certainty, because other diseases (e.g., migraine) can cause the same constellation of symptoms and because there is no 'gold standard' diagnostic test to definitively identify or exclude MD (Thorp, Shehab et al. 2003; Kim, Wiet et al. 2005; Thirlwall and Kundu 2006).

The estimated incidence of MD in persons of European descent is 1 per 1000, and in persons of Japanese descent, 0.03-0.37 per 1000 (Morrison 1995; Watanabe, Mizukoshi et al. 1995; Shojaku and Watanabe 1997). An estimated 100-200 new diagnoses per million population are made each year (Thirlwall and Kundu 2006), with 30-50% of these persons developing bilateral symptoms within two years of presenting with unilateral symptoms (Sajjadi 2002). Of these symptoms, the vertigo can be especially debilitating and can severely impact many activities of daily living (Mancini, Catalani et al. 2002).

The hallmark histopathologic feature of MD is endolymphatic hydrops, a descriptive term for the outward 'ballooning' of the endolymphatic compartment of the membranous labyrinth seen on temporal bone histopathology. Although hydrops has been interpreted to reflect a pressure gradient between the scala media and scala vestibuli, (Andrews 2004), it is not known whether hydrops is a consequence of abnormal endolymph production or absorption (Mancini, Catalani et al. 2002). Hydrops is also not unique to MD and can be seen with head trauma, mumps infection, otosyphilis, Cogan's syndrome and labyrinth neoplasms (1995).

Several studies have attempted to identify genetic factors important in MD. One approach has been to select and sequence candidate genes based on putative function. Included in this list are coagulation factor C homology (*COCH*), *ATQ1* (antiquitin) and *AQP2* (aquaporin 2) (Morrison, Mowbray et al. 1994; 1995; Lynch, Cameron et al. 2002; Mhatre, Jero et al. 2002; Paparella and Djalilian 2002; Usami, Takahashi et al. 2003;

Sanchez, Lopez-Escamez et al. 2004). Studies have also looked at HLA associations (Xenellis, Morrison et al. 1986; Koyama, Mitsuishi et al. 1993; Morrison, Mowbray et al. 1994; Arweiler, Jahnke et al. 1995; Yeo, Park et al. 2002; Koo, Oh et al. 2003). To date, however, no disease-causing mutations have been identified in any gene that segregates with the MD phenotype, which is not surprising given the sporadic nature of most cases of MD.

An alternative approach is to consider MD as a complex disease and use a case-control study design to identify associations between specific genetic variants and MD (Tabor, Risch et al. 2002). Association studies can be done on a gene-by-gene basis or across the entire genome (genome-wide association, GWA). Using the former approach, Doi and colleagues have reported that two single nucleotide polymorphisms (SNPs) in *KCNE1* (21q22.12) and *KCNE3* (11q13.4) are associated with MD in Japanese MD patients (Doi, Sato et al. 2005). These genes are members of the *KCNE* gene family and encode the accessory MinK proteins, MinK and MiRP2. MinK, encoded by *KCNE1*, associates with KCNQ1 and produces the I_{KS} current (Abbott and Goldstein 1998; Melman, Krummerman et al. 2002), while MiRP2, encoded by *KCNE3*, associates with Kv3.4 (Melman, Krummerman et al. 2002).

In the inner ear, *KCNE1* is expressed at the apical membrane of vestibular dark cells and at the apical surface of marginal cells (Sakagami, Fukazawa et al. 1991; Mori, Sakagami et al. 1993; Wangemann, Liu et al. 1995; Nicolas, Dememes et al. 2001), while *KCNE3* is found in the epithelium of the distal portion of the endolymphatic sac (Doi, Sato et al. 2005). Based on these expression patterns and the role MinK and MiRP2 play in ion transport, Doi and colleagues hypothesized that these two genes are important in inner ear homeostasis and therefore may play a role in MD. Their results support this hypothesis.

A major limitation of association studies is a false-positive association. To accept a positive association as valid, it is widely recommended that results be replicated in

other populations (Tabor, Risch et al. 2002). The purpose of this study was to replicate the association reported by Doi et al in a group of Caucasian MD patients accrued in the United States.

Materials and Methods

MD Patients & Controls

One-hundred-eighty unrelated Caucasian, non-Hispanic individuals diagnosed with definitive MD based on AAO-HNS criteria (1995) were ascertained under IRB-approved guidelines from the University of Iowa and Johns Hopkins University (US MD). The diagnosis of definitive MD is exclusionary and requires documentation of at least two episodes of vertigo lasting a minimum of 20 minutes, documented hearing loss on at least one occasion, and tinnitus or aural fullness in the affected ear (1995). Genomic DNA was extracted from peripheral blood using established techniques (Grimberg, Nawoschik et al. 1989). One-hundred-eighty unrelated individuals matched for age (55.6% within 2 years; 40.5% within 3-5 years), race, ethnicity and gender were used as controls (US CTRLS). Patients over 70 years old were matched with controls over 70 years of age (3.9%). The Caucasian MD patient group consisted of 78 males (46%) and 102 females (54%) ranging in age from 22.9-83.5 years (average age, 54.3 years) (US MD). One patient has a known family history of MD, the remaining are sporadic cases.

We obtained a Japanese control group ascertained from Okayama University (*n*=134, *KCNE1*; *n*=131, *KCNE3*) (Okayama CTRLS). Allele frequencies for the two SNPs studied by Doi et al., rs1805127 (*KCNE1*) and rs2270676 (*KCNE3*) were obtained from the HapMap Japanese in Tokyo (HapMap JPT) and Utah residents with ancestry from northern and western Europe (HapMap CEPH) (2003). The Institutional Review Boards of the University of Iowa and the Johns Hopkins University approved all procedures.

Population Substructure Analysis

Population stratification within our patients and matched controls was excluded by: (1) genotyping the *LCT* promoter SNP -13910 C→T (rs4988235) (Campbell, Ogburn et al. 2005) (Table 3); (2) genotyping 26 microsatellite markers distributed throughout the genome (Pritchard and Rosenberg 1999). The χ^2 test statistic was calculated for each marker (Table 4).

Polymorphism Detection and Gene Screening

Intronic primers were selected with Primer3 (http://frodo.wi.mit.edu/) to amplify the coding regions and surrounding intronic boundaries of *KCNE1* (NM_000219) and *KCNE3* (NM_005472) (Rozen and Skaletsky 2000). Following polymerase chain reaction (PCR) amplification, bidirectional sequencing was performed with BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) on an ABI 3730 Sequencer (Applied Biosystems, Foster City, CA). Sequences were analyzed and allele frequencies calculated for previously reported and novel variants in all patients and controls.

Statistical Analysis

Differences in frequencies were compared by Chi-square tests of independence (Preacher 2001), with p-values ≤0.05 considered significant. A Yate's correction for continuity was used to improve the accuracy of the null condition sampling distribution when an expected frequency was <1 or < 5 in at least 20% of cells. Deviations from Hardy-Weinberg equilibrium (HWE) were calculated as previously described (Sasieni 1997). Haplotypes were created and analyzed with Haploview (v.3.32) (http://www.broad.mit.edu/mpg/haploview/) (Barrett, Fry et al. 2005).

Table 3. Allele and genotype frequencies for *LCT* promoter SNP rs4988235 (Campbell, Ogburn et al. 2005).

A. Genotype and allele frequencies for rs4988235						
	TT	TC	CC	T	C	Total
US MD	65 (36.1%)	81 (45.0%)	34 (18.9%)	211 (58.6%)	149 (41.4%)	180
US CTRLS	66 (36.7%)	90 (50.0%)	24 (13.3%)	222 (61.7%)	138 (38.3%)	180

B. Iowa MD and Controls *LCT* rs4988235 Results												
Alleles (US MD)		Alleles (US CTRLS)		χ^2 p-value	Genotypes (US MD)			Genotypes (US CTRLS)			χ^2 p-value	*HWE p-value (US CTRLS)
211(T)	149 (C)	222 (T)	139 (C)	0.402	65 (TT)	81 (TC)	34 (CC)	66 (TT)	90 (TC)	24 (CC)	0.332	0.860

*HWE = Hardy-Weinberg equilibrium

Table 4. Microsatellite marker results for population substructure analysis on Caucasian MD patients and matched controls. A Pearson χ^2 p-value ≤0.05 was considered significant.

Marker	Chromosome	% Genotyped	χ2 p-value
D1S199	1p36.13	99.4%	0.150
D1S2836	1q44	98.9%	0.814
D2S305	2p24.1	98.9%	0.817
D2S151	2q22.3	99.4%	0.541
D3S1297	3p26.3	99.4%	0.892
D4S415	4q34.3	98.9%	0.888
D5S436	5q32	98.9%	0.261
D6S460	6q14.1	99.4%	0.370
D7S493	7p15.3	99.2%	0.820
D8S549	8p22	99.4%	0.029
D9S283	9q22.2	99.4%	0.428
D10S197	10p12.1	99.7%	0.677
D10S185	10q23.33	99.4%	0.335
D11S937	11q14.1	99.2%	0.479
D12S352	12p13.33	99.2%	0.324
D13S263	13q14.11	99.4%	0.696
D14S275	14q12	99.7%	0.262
D15S128	15q11.2	98.60%	0.731
D16S520	16q24.1	99.7%	0.797
D17S831	17p13.3	99.2%	0.410
D18S452	18p11.31	99.2%	0.782
D18S478	18q12.1	99.7%	0.756
D19S220	19q13.13	99.7%	0.084
D20S119	20q13.12	99.4%	0.392
D21S1912	21q22.3	98.9%	0.802
D22S423	22q13.1	97.8%	0.297
	averages:	99.2%	0.532

Results

KCNE3

In our Caucasian MD patient population, for rs2270676, 80.0% of patients were homozygous for the T allele (*n*=144), 1.1% of patients were homozygous for the C allele (*n*=2), and 18.9% of patients were heterozygous (*n*=34) (Table 5A). We found no difference in allele or genotype frequencies for this SNP between Caucasian MD patients and matched controls (χ^2 p-value= 0.635 and 0.870 for allele and genotype, respectively) (Table 5B). No difference was observed (χ^2 p-value= 0.862 and 0.129 for allele and genotype, respectively) between patient groups, indicating that our inability to replicate the association was not due to differences between MD patients in this study and MD patients in the Doi et al study (Table 5C). Comparison of both our MD patients and our matched controls to the HapMap CEPH Caucasian controls revealed no differences (Tables 5B and 5D), however our controls do differ significantly from the controls used in the Doi et al study (χ^2 p-value= 4.9×10^{-6} for allele and 4.0×10^{-8} for genotype, respectively) (Table 5D).

Comparison of the Doi MD population to our Okayama controls showed a weaker but still significant association for genotype frequency (χ^2 p-value= 0.025), and with HapMap JPT controls, the association was no longer significant (Table 5B). A significant difference was found in allele and genotype frequencies when Doi controls were compared to both Okayama controls and HapMap JPT data, while comparison of Okayama controls to HapMap JPT data showed no difference between allele and genotype frequencies (Table 5D). The Hardy-Weinberg equilibrium p-value for Doi controls was significant (p=0.035) (Table 5A). No significant associations between our MD patients and matched controls were found for the other six SNPs identified in *KCNE3* (Table 6), and Haploview analysis did not reveal any significant haplotypes in these populations (data not shown).

Table 5. Association study results for rs2270676 in *KCNE3*. *HWE = Hardy-Weinberg equilibrium; P-values <0.05 indicated in bold.

A. Allele and genotype frequencies for rs2270676 in all populations

	TT	TC	CC	*HWE p-value	T	C	Total
US MD	144 (80.0%)	34 (18.9%)	2 (1.1%)	1.000	322 (89.4%)	38 (10.6%)	180
US CTRLS	140 (77.8%)	38 (21.1%)	2 (1.1%)	0.972	318 (88.3%)	42 (11.7%)	180
HAPMAP CEU	47 (78.3%)	12 (20.0%)	1 (1.7%)	0.988	106 (88.3%)	14 (11.7%)	60
DOI JAPANESE MD	52 (82.5%)	8 (12.7%)	3 (4.8%)	0.313	112 (89.9%)	14 (11.1%)	63
DOI JAPANESE CTRLS	196 (95.6%)	5 (2.4%)	4 (2.0%)	**0.035**	397 (96.8%)	13 (3.2%)	205
OKAYAMA CTRLS	87 (66.4%)	40 (30.5%)	4 (3.1%)	0.986	214 (81.7%)	48 (18.3%)	131
HAPMAP JPT CTRLS	31 (68.9%)	10 (22.2%)	4 (8.9%)	0.426	72 (80.0%)	18 (20.0%)	45

B. Patients vs. Controls for rs2270676 in *KCNE3*

Ménière	Controls	Allele χ^2 p-value	Genotype χ^2 p-value
US MD VS	US CTRLS	0.635	0.870
	HAPMAP CEU	0.735	0.925
DOI JAPANESE MD VS	DOI JAPANESE CTRLS	$\mathbf{3.7x10^{-4}}$	$\mathbf{1.7x10^{-3}}$
	OKAYAMA CTRLS	0.070	**0.025**
	HAPMAP JPT CTRLS	0.070	0.252

Table 5-continued.

C. Disease vs. Disease for rs2270676			
Ménière 1	Ménière 2	Allele χ^2 p-value	Genotype χ^2 p-value
US MD VS	DOI JAPANESE MD	0.862	0.129

D. Controls vs. Controls for rs2270676			
Controls 1	Controls 2	Allele χ^2 p-value	Genotype χ^2 p-value
US CTRLS VS	DOI JAPANESE CTRLS	**4.9×10^{-6}**	**4.0×10^{-8}**
	OKAYAMA CTRLS	**0.020**	0.064
	HAPMAP CEU	1.000	0.932
	HAPMAP JPT CTRLS	**0.038**	**0.014**
DOI JAPANESE CTRLS VS	OKAYAMA CTRLS	**$<1 \times 10^{-8}$**	**$<1 \times 10^{-8}$**
	HAPMAP JPT CTRLS	**$<1 \times 10^{-8}$**	**9.0×10^{-8}**
OKAYAMA CTRLS VS	HAPMAP JPT CTRLS	0.725	0.187

Table 6. Genotype frequencies for *KCNE3* in Caucasian MD patients and matched controls. A Yate's χ^2 p-value was used when n<5. The SNP in bold, rs2270676, is the SNP previously reported by Doi et al (Doi, Sato et al. 2005). *HWE = Hardy-Weinberg equilibrium.

SNP	Amino Acid Change	Genotypes (US MD)			Genotypes (US CTRLS)			Genotype χ^2 p-value	*HWE (US CTRLS) p-value	Allele χ^2 p-value
rs17215444	R47G	180 (CC)	0 (CG)	0 (GG)	180 (CC)	0 (CG)	0 (GG)	NA	NA	NA
c.194T>C	M65T	180 (TT)	0 (TC)	0 (CC)	179 (TT)	1 (TC)	0 (CC)	1.00	0.999	0.317
rs2270676	F66F	144 (TT)	34 (TC)	2 (CC)	140 (TT)	38 (TC)	2 (CC)	0.870	0.972	0.635
rs17221826	I76I	180 (CC)	0 (CT)	0 (TT)	180 (CC)	0 (CT)	0 (TT)	NA	NA	NA
rs17215437	R83H	180 (GG)	0 (GA)	0 (AA)	179 (GG)	1 (GA)	0 (AA)	1.00	0.999	0.317
rs17221833	R88H	180 (GG)	0 (GA)	0 (AA)	180 (GG)	0 (GA)	0 (AA)	NA	NA	NA
rs11822977	NA	180 (CC)	0 (CT)	0 (TT)	180 (CC)	0 (CT)	0 (TT)	NA	NA	NA

KCNE1

In our MD population, for rs1805127, 42.8% of patients were homozygous for the G allele (*n*=77) and 12.2% of patients were homozygous for the A allele (*n*=22), and 45.0% of patients were heterozygous (*n*= 81), and no differences in allele or genotype frequencies were seen when we compared this group to our controls (χ^2 p-value=0.394 and 0.552, respectively) (Tables 7A and 7B). Comparison of allele and genotype frequencies between Doi MD and our MD patients also showed no differences (χ^2 p-value=0.903 and 0.957, respectively) (Table 7C). While our controls did differ from the Doi controls, there were no differences in allele or genotype frequencies between our controls and HapMap CEPH Caucasian data or between our MD patients and HapMap CEPH Caucasian data (Table 7D and 7B).

Comparison between Doi MD patients and Okayama controls did identify a significant difference; however, it was weaker than reported by Doi, and there no association between Doi MD patients and HapMap JPT data (Table 7B). There were significant differences between Doi controls and Okayama controls, and also between Doi controls and HapMap JPT data, however there were no differences between Okayama controls and HapMap JPT data (Table 7D). The Hardy-Weinberg equilibrium p-value for the Doi controls was significant ($p=1.33x10^{-5}$) (Table 7A). No significant associations between our MD patients and matched controls were found for the other six SNPs identified in *KCNE1* (Table 8), and Haploview analysis did not reveal any significant haplotypes in these populations (data not shown).

Table 7. Association study results for rs1805127 in *KCNE1*. *HWE = Hardy-Weinberg equilibrium; P-values <0.05 indicated in bold.

A. Allele and genotype frequencies for rs1805127 in all populations

	GG	AG	AA	*HWE p-value	G	A	Total
US MD	77 (42.8%)	81 (45.0%)	22 (12.2%)	0.998	235 (65.3%)	125 (34.7%)	180
US CTRLS	67 (37.2%)	90 (50.0%)	23 (12.8%)	0.832	224 (62.2%)	136 (37.8%)	180
HAPMAP CEU	21 (35.0%)	32 (53.3%)	7 (11.7%)	0.778	74 (61.7%)	46 (38.3%)	60
DOI JAPANESE MD	28 (44.4%)	27 (42.9%)	8 (12.7%)	0.966	83 (65.9%)	43 (34.1%)	63
DOI JAPANESE CTRLS	192 (81.0%)	25 (10.6%)	20 (8.4%)	**1.33×10^{-5}**	409 (86.3%)	65 (13.7%)	237
OKAYAMA CTRLS	73 (54.5%)	57 (42.5%)	4 (3.0%)	0.380	203 (75.7%)	65 (24.3%)	134
HAPMAP JPT CTRLS	22 (50.0%)	18 (40.9%)	4 (9.1%)	0.997	62 (70.4%)	26 (29.6%)	44

B. Patients vs. Controls for rs1805127

Ménière	Controls	Allele χ^2 p-value	Genotype χ^2 p-value
US MD VS	US CTRLS	0.394	0.552
	HAPMAP CEU	0.474	0.511
DOI JAPANESE MD VS	DOI JAPANESE CTRLS	**1.1×10^{-7}**	**$<1 \times 10^{-8}$**
	OKAYAMA CTRLS	**0.040**	**0.024**
	HAPMAP JPT CTRLS	0.480	0.781

Table 7-continued.

C. Disease vs. Disease for rs1805127			
Ménière 1	Ménière 2	Allele χ^2 p-value	Genotype χ^2 p-value
US MD VS	DOI JAPANESE MD	0.903	0.957

D. Controls vs. Controls for rs1805127			
Controls 1	Controls 2	Allele χ^2 p-value	Genotype χ^2 p-value
US CTRLS VS	DOI JAPANESE CTRLS	**$<1x10^{-8}$**	**$<1x10^{-8}$**
	OKAYAMA CTRLS	**$3.3x10^{-4}$**	**$6.7x10^{-4}$**
	HAPMAP CEU	0.913	0.903
	HAPMAP JPT CTRLS	0.150	0.293
DOI JAPANESE CTRLS VS	OKAYAMA CTRLS	**$2.9x10^{-4}$**	**$<1x10^{-8}$**
	HAPMAP JPT CTRLS	**$2.1x10^{-4}$**	**$1.3x10^{-6}$**
OKAYAMA CTRLS VS	HAPMAP JPT CTRLS	0.323	0.235

Table 8. Allele frequencies & genotype frequencies for *KCNE1* in Caucasian MD patients and matched controls. A Yate's $\chi 2$ p-value was used when $n<5$. The SNP in bold, rs1805127, is the SNP previously reported by Doi et al (Doi, Sato et al. 2005). *HWE= Hardy-Weinberg equilibrium.

SNP	Amino Acid Change	Genotypes (US MD)			Genotypes (US CTRLS)			Genotype χ^2 p-value	*HWE (US CTRLS) p-value	Allele χ^2 p-value
rs28933384	I7T	180 (CC)	0 (CT)	0 (TT)	180 (CC)	0 (CT)	0 (TT)	NA	NA	NA
rs17173510	S28S	179 (GG)	1 (AG)	0 (AA)	178 (GG)	2 (AG)	0 (AA)	1.00	0.997	1.00
rs17857111	H32R	180 (GG)	0 (AG)	0 (AA)	180 (GG)	0 (AG)	0 (AA)	0.870	NA	NA
rs1805127	S38G	77 (GG)	81 (AG)	22 (AA)	67 (GG)	90 (AG)	23 (AA)	0.552	0.832	0.394
rs17173509	G52A	180 (GG)	0 (CG)	0 (CC)	180 (GG)	0 (CG)	0 (CC)	1.00	NA	1.00
rs17173508	F54F	180 (CC)	0 (CT)	0 (TT)	180 (CC)	0 (CT)	0 (TT)	1.00	NA	1.00
rs1805128	D85N	179 (GG)	1 (AG)	0 (AA)	177 (GG)	3 (AG)	0 (AA)	0.616	0.994	0.616

Discussion

Candidate-gene association studies are frequently used as a first step in identifying potential disease pathways. This approach presumes an understanding of disease pathogenesis to select a 'good' candidate gene and requires well-defined matched case and control populations to generate robust data. The selection of a 'good' candidate gene for MD is problematic since our understanding of its etiology and pathophysiology is marginal and naturally occurring animal models do not exist (Tabor, Risch et al. 2002). Not surprisingly, there have been no MD case-control candidate gene studies that have identified any significant genetic associations across multiple populations.

Follow-up studies are often unable to replicate the results of an initial association study, or the association is not as strong as originally reported, for several possible reasons (Tabor, Risch et al. 2002). First, disease etiology may differ among ethnic groups. Second, study design can lead to a lack of reproducibility if the phenotype is not carefully defined in the case populations. Third, problems with sample collection can result in population stratification in the case or control population. Fourth, ethnic-specific variants may exist necessitating analysis of the entire gene to avoid missing associations in other populations (Chanock, Manolio et al. 2007). Finally, the previous associations could be spurious (Lander and Schork 1994; Tabor, Risch et al. 2002).

Doi et al report two SNPs that are associated with MD in the Japanese population: rs2270676, a synonymous SNP (F66F) in *KCNE3*, and rs1805127, a nonsynonymous SNP (S38G) in *KCNE1* ($p=0.0015$ and $p<0.001$, respectively) (Berezin, Glaser et al. 2004; Doi, Sato et al. 2005). We were unable to replicate these associations. In addition, when our MD patients were compared to HapMap CEPH Caucasian data, we also observed no difference in allele or genotype frequencies for either SNP. To determine why these associations failed to replicate, we compared Doi MD allele and genotype frequencies to our MD population and found no differences for either SNP in these

patient groups, suggesting that our inability to replicate the association was not due to differences between patient groups.

To verify whether our control group was appropriate, we compared our controls to HapMap CEPH Caucasian data and found no differences for either SNP. However, our controls did differ significantly from the Doi controls for both SNPs. Further comparisons between Doi MD patients and various control groups showed that when Okayama controls are used, the significance of the associations is less robust and when HapMap JPT data are used, the associations disappear. These data suggest that the Doi control group is not an appropriate control group for this study. Consistent with this possibility, there are significant differences between allele frequencies in the Doi controls and both HapMap JPT data and Okayama controls, suggesting that the Doi controls are not suitable for detecting a true association in Doi MD patients.

Deviation from Hardy-Weinberg equilibrium (HWE) can indicate genotyping errors or population admixture (Tiret and Cambien 1995; Salanti, Amountza et al. 2005). Significant HWE p-values for Doi controls suggests the association could be spurious as a result of genotyping errors or population admixture (Tiret and Cambien 1995; Salanti, Amountza et al. 2005). Population stratification occurs if disease frequency varies with ethnicity, and a marker found at a high frequency in one ethnic group may have a positive association with the disease phenotype even if it is not the causative allele or near a causative allele (Tsai, Choudhry et al. 2005). Doi et al did not indicate the origin of the patients or controls or whether the two populations were geographically matched. Population substructure in the Japanese has been previously reported and if the Doi MD patients and controls were from different regions of Japan, population substructure could exist and lead to a spurious association (Yamaguchi-Kabata, Nakazono et al. 2008). Of note, Doi did not state if population substructure analysis was performed.

Our study groups passed two population substructure analyses and controls were in HWE, therefore based on our data we conclude that neither SNP is associated with

MD. To determine if either SNP was in linkage disequilibrium with a disease-causing variant, we also sequenced the coding regions of both genes in all patients and controls. No variants were identified that differed significantly in either genotype or haplotype between our MD patients and matched controls (Tables 6 and 8), suggesting these genes are not associated with MD. It is difficult to compare haploblock structure between populations as haplotype boundaries have been shown to vary with populations of different ancestry (Cardon and Abecasis 2003; Liu, Sawyer et al. 2004). However, as the allele frequencies between the Doi MD and US MD populations did not differ, we do not anticipate a causal variant in linkage disequilibrium with the SNPs reported by Doi and colleagues.

In addition to population substructure stratification in the control group as a source of error, spurious associations can also result from small sample sizes (Ioannidis, Ntzani et al. 2001) and it is worth noting that our sample size is larger than the sample size used by Doi. It should also be recognized that both of these studies are important, as replication of an association study is a critical step to complete before exploring functional studies to determine the pathophysiological basis of a genetic association. Our inability to replicate Doi's associations suggests that *KCNE1* and *KCNE3* do not have a role in MD and that genetic testing for variants in *KCNE1* and *KCNE3* in patients with MD is not warranted.

Acknowledgements

The authors sincerely thank all of the patients and individuals who served as controls for their participation in this study. We would like to thank Kathy Williams at the University of Iowa for her help in enrolling patients and collecting samples. No authors in this study report a conflict of interest. This work was supported in part by a grant from the American Otological Society to RJHS.

Replication of Spanish MD and iNOS association study

Introduction

A potential collaborator requested we attempt to replicate his unpublished finding that a promoter polymorphism of *iNOS* (inducible nitric oxide synthase; NOS2A; 17q11.1) is associated with MD in a Spanish population. It is believed that *iNOS* is involved in an immune mediated response. The theory of an autoimmune component to MD prompted the collaborator to select this gene for the candidate gene association study.

Materials and Methods

Primers from the previous study (Lopez-Escamez 2009) were used for amplification with one modification: to the forward primer, an M13 tail was added. The test cohort mentioned above was analyzed for the variant. PCR products were run on a 3130 XL Genetic Analyzer (AB), and GeneMapper v4.0 was used to assign alleles for all patients and controls. Bidirectional sequencing was performed on a subset of patients to confirm the number of repeats for accurate genotype assignment. Differences in allele and genotype frequencies were compared and p-values ≤0.05 considered significant.

Results

Overall, our results indicated the association was in the opposite direction of the original study. In addition, comparison of the allele frequencies for the patients and controls in the current study resulted in a χ^2 p-value of 0.7261 indicating no difference in allele frequencies for this polymorphism between Caucasian MD patients and matched controls.

Discussion

The initial study had a high percentage of bilateral MD cases which is unusual and suggestive of questionable diagnosis. Therefore, one reason we may not have been

able to replicate the initial study could be due to poor definition of the patient cohort. The collaborator mentioned controls were matched based on HLA status which is not standard practice. Therefore, another reason we may not have replicated the initial study could be due to inappropriate controls in the initial study leading to a spurious result. Alternatively, there could be differences between ethnic groups, we have not investigated this possibility. No further work is being completed on this project.

CHAPTER III

AQP4 AND SYNDROMIC MENIERE'S DISEASE

Abstract

Ménière's disease (MD) is a complex idiopathic disorder of the inner ear characterized by hearing loss, tinnitus, and vertigo. The exclusionary diagnosis is difficult to make and requires two or more attacks of vertigo lasting more than 20 minutes, hearing loss and tinnitus or aural fullness documented (1995). Endolymphatic hydrops is the histopathologic feature which is a swelling of one compartment of the inner ear. Majority of cases are sporadic although occasionally MD segregates in an autosomal dominant manner in families with 60-90% penetrance (Morrison 1995; Frykholm, Larsen et al. 2006). Migraine has also been noted to segregate with MD in some families suggestive of a syndromic form of MD. MD is a complex disease and to date a genetic component has not been identified across populations responsible for sporadic, familial, or syndromic disease (Chapter 1).

Aquaporin 4 (*AQP4*) is a member of the aquaporin family and is found to be expressed in multiple cell types in the cochlea and vestibular systems of the inner ear. The function of AQP4 as a water channel suggests it may play a role in maintaining inner ear homeostasis, specifically endolymph homeostasis. In this chapter we will test the hypothesis that a candidate gene association study will identify variants in *AQP4* associated with MD which result in an osmotic imbalance in the inner ear leading to endolymphatic hydrops.

Introduction

The purpose of this study was to determine if variants in *AQP4* (chromosome 18q11.2-12.1) are associated with Ménière's disease. Although the aquaporin family of proteins are abundantly expressed in the cochlea and vestibular systems of the inner ear, to date none of the aquaporins has been associated with ear disease in humans. We chose

to study *AQP4* as multiple studies have demonstrated expression of aquaporin 4 in the ear (Takumi, Nagelhus et al. 1998; Beitz, Kumagami et al. 1999; Li and Verkman 2001; Minami, Kobayashi et al. 2001; Fukushima, Kitahara et al. 2002; Zhong and Liu 2003; Andrews 2004), and the null mice have hearing loss (Li and Verkman 2001) (Figure 1).

The aquaporins are small hydrophobic, integral membrane pores which actively transport water into and out of cells through a regulated process requiring an extremely low amount of energy due to existing osmotic gradients. The center of the pore has hydrogen bonds which allow the selective movement of water molecules single file through the cell (Andrews 2004). The aquaporins (AQPs) have a characteristic 'hourglass' structure, and two signature sequence motifs of NPA (Asn-Pro-Ala), one in the amino half, and the other in the carboxy-terminal half of the gene (King, Kozono et al. 2004). To date the AQPs have been found to be widely expressed in most animals and plants, with thirteen AQPs identified to date in mammals (Verkman 2005). Aquaporins are especially important in tissues such as the eye, lung, brain, and kidney which have a high demand for water transport, and also have important roles in osmoregulation (Zhong and Liu 2003; Couloigner, Berrebi et al. 2004). In general, the AQPs are constitutively found at the plasma membrane as tetramers and are regulated at the transcriptional-level.

Mutations in several *AQPs* have been found to cause disease in humans; *AQP2* mutations have been identified in individuals with hereditary nephrogenic diabetes insipidus (NDI) (King, Kozono et al. 2004). Individuals with AQP1 deficiency have a urinary concentrating defect (King, Choi et al. 2001). Some individuals with autosomal dominant congenital cataracts have *AQP0* mutations (Berry, Francis et al. 2000), and some patients with Sjogren's syndrome have decreased AQP5 in the apical membrane of salivary-gland secretory cells (Steinfeld, Cogan et al. 2001; Tsubota, Hirai et al. 2001).

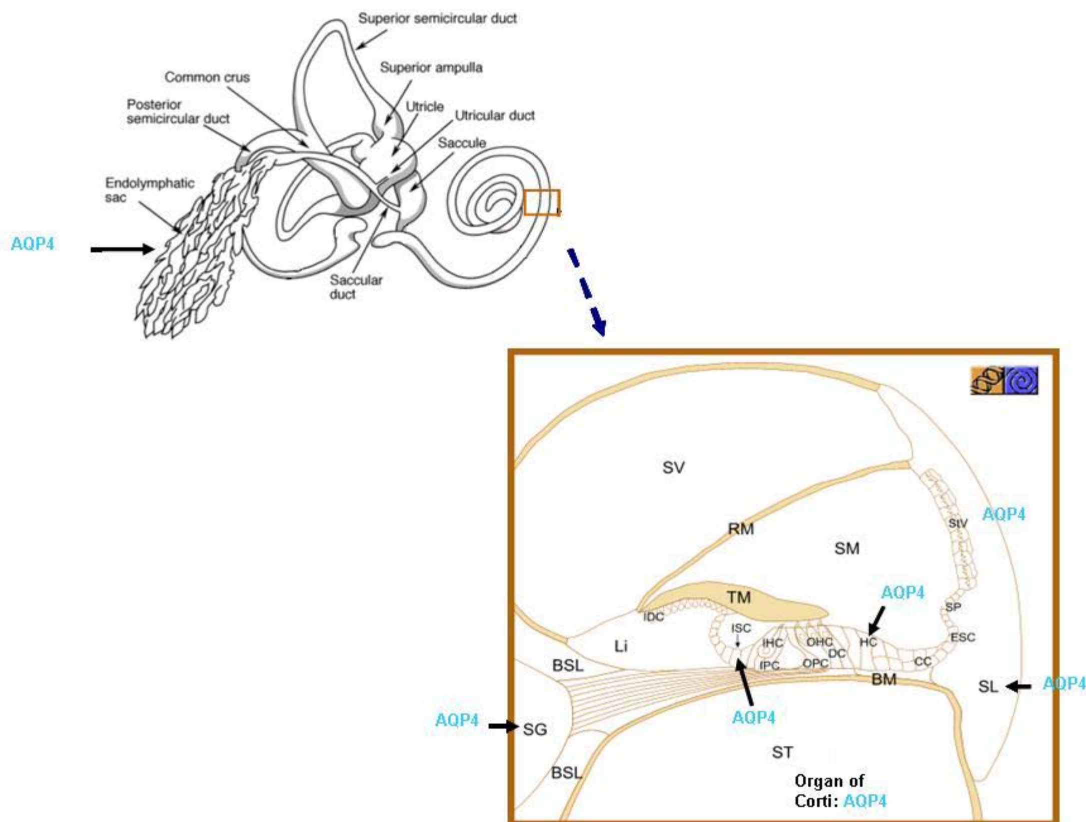
Several AQPs are found in the inner ear (Figure 1). AQP4 has been found to be expressed in the supporting cells, Hensen's cells, inner sulcus cells, and lateral and basal membrane of the ciliated cells of the rat cochlea (Takumi, Nagelhus et al. 1998; Minami,

Kobayashi et al. 2001; Fukushima, Kitahara et al. 2002), as well as the supporting cells of the rat vestibular end organs (Takumi, Nagelhus et al. 1998; Beitz, Kumagami et al. 1999; Fukushima, Kitahara et al. 2002). AQP4 is expressed in the epithelial cells and subepithelial cells of the guinea pig endolymphatic sac, as well as the stria vascularis, spiral ligament, spiral ganglion, and organ of Corti in the guinea pig cochlea (Zhong and Liu 2003). Due to the cellular expression of AQP4 in the cochlea and vestibular system, AQP4 may have an important role in endolymph and perilymph homeostasis in the inner ear (Mhatre, Jero et al. 2002; Andrews 2004).

Mice deficient for *Aqp4* have varying degrees of hearing loss depending on their genetic background (Li and Verkman 2001; Mhatre, Stern et al. 2002). Studies have shown that if vasopressin is administered to guinea pigs, after one week prominent endolymphatic hydrops develops, suggesting a role for vasopressin in the development of endolymphatic hydrops (Takeda, Takeda et al. 2000). Overall, the aquaporins may play a role in regulation of endolymphatic fluid, and would be good candidate genes for susceptibility to MD.

In this study, we will test the hypothesis that MD is caused by an osmotic imbalance in the inner ear associated with functional differences of allele variants of *AQP4*. Although the initial trigger(s) of Ménière’s disease are unknown, we hypothesize the allele variants of the *AQP4* allow a permissive background for which endolymphatic hydrops to develop, by operating as a molecular valve to regulate rapid water movement across cell membranes.

Figure 1. Aquaporin expression in the inner ear. BM= Basilar membrane; BSL= Bony spiral lamina; CC= Claudius cells; DC= Deiters' cells; ESC= External sulcus cells; HC= Hensen cells; IDC= Interdental cells; IHC = Inner hair cell; IPC= Inner pillar cell; ISC= Inner sulcus cells; Li= Limbus; OHC= Outer hair cells; OPC= Outer pillar cell; RM= Reissners' membrane; SG= Spiral ganglion; SL= Spiral ligament; SM= Scala media; SP= Spiral prominence; ST= Scala tympani; StV= Stria vascularis; SV= Scala vestibuli; TM= Tectorial membrane. Figure modified from (Ballenger 2003; Van Camp G 2004). (Takumi, Nagelhus et al. 1998; Beitz, Kumagami et al. 1999; Mhatre, Steinbach et al. 1999; Fukushima, Kitahara et al. 2002; Huang, Chen et al. 2002; Mhatre, Jero et al. 2002; Ballenger 2003; Beitz, Zenner et al. 2003; Sawada, Takeda et al. 2003; Zhong and Liu 2003; Couloigner, Berrebi et al. 2004; Van Camp G 2004).



Materials and Methods

Subjects

Genomic DNA was extracted from peripheral blood of 124 unrelated Caucasian MD patients (AAO-HNS 1995) as well as 248 controls matched for gender, age, and ethnicity were under IRB-approved guidelines from the University of Iowa and Johns Hopkins University (Grimberg, Nawoschik et al. 1989; 1995). Population substructure was ruled out after genotyping the *LCT* promoter SNP rs4988235, with a genotype χ^2 p-value of 0.1967, allelic χ^2 p-value of 0.2142 (Table 9) (Campbell, Ogburn et al. 2005). A replication cohort of 124 Caucasian MD patients (AAO-HNS 1995) matched to the test cohort for gender, age and ethnicity along with 228 controls (matched for gender, age, and ethnicity) were genotyped for the single nucleotide variant (SNV), M224T by Sanger sequencing.

In addition, 1051 controls from the HGDP-CEPH Human Genome Diversity Cell Line Panel were genotyped for the SNV, M224T (Cann, de Toma et al. 2002). Detailed medical histories and DNA was obtained from the family members of two probands (UIMEN050-A, UIMEN078-A), and screened for M224T.

Candidate Gene Screen

Primers were selected with Primer3 (http://frodo.wi.mit.edu/) to amplify the coding regions and surrounding intronic boundaries and 3'UTR of *AQP4* (NM_001650) (Rozen and Skaletsky 2000). Polymerase chain reaction (PCR) amplification, followed by sequencing with BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) was performed on an ABI 3730 Sequencer (Applied Biosystems, Foster City, CA) on all patients and controls.

Previously reported and novel SNVs were genotyped throughout the gene and allele frequencies compared between patients and controls using a χ^2 test statistic with significance of $p<0.05$. Haploblocks were analyzed with Haploview (v.3.32,

http://www.broad.mit.edu/mpg/haploview/) for whole gene associations (Barrett, Fry et al. 2005).

Short Tandem Repeat Polymorphism (STRP) Analysis

Repeat polymorphisms were identified in the region surrounding *AQP4* and fluorescently labeled primers designed with Primer3 (http://frodo.wi.mit.edu/) to amplify the repeats in all family members. Amplification further described in Appendix C. GeneMapper v4.0 was used to assign alleles, and haplotypes were manually reconstructed.

Results

Subjects

Detailed medical history from families UIMEN050 and UIMEN078 revealed additional phenotypes present in both families, and some individuals may have partial MD (Tables 10 and 11, Figures 2 and 3). Individuals in family UIMEN050 report their ethnicity as German. Individuals in family UIMEN078 report their ethnicity as German, Scottish and Native American.

Candidate Gene Screen

Thirteen previously reported variants and 10 novel variants were genotyped in all patients and all controls (Table 12). A novel coding variant, M224T was identified in two patients (UIMEN050-A and UIMEN078-A). The M224T variant was not identified in 424 matched control individuals (848 control chromosomes), but was found in 2 of 1051 CEPH Diversity control individuals. There was not an association between haploblocks and *AQP4* in the test cohort (Figure 4).

Short Tandem Repeat Polymorphism (STRP) Analysis

Chromosome 18 haplotypes revealed families UIMEN050 and UIMEN078 may share a small region of autozygosity by descent surround M224T (Figures 5 and 6).

Table 9. Allele and genotype frequencies for *LCT* promoter SNP rs4988235 (Campbell, Ogburn et al. 2005). *HWE = Hardy-Weinberg equilibrium.

A. Genotype and allele frequencies for rs4988235						
	TT	TC	CC	T	C	Total
MD	50 (41.0%)	49 (40.0%)	23 (19.0%)	149 (61.1%)	95 (38.9%)	122
CTRLS	99 (43.4%)	102 (44.7%)	27 (11.9%)	300 (65.8%)	156 (34.2%)	228

B. Iowa MD and Controls *LCT* rs4988235 Results						
Alleles (MD)	Alleles (CTRLS)	χ^2 p-value	Genotypes (MD)	Genotypes (CTRLS)	χ^2 p-value	*HWE p-value (CTRLS)
149 (T) 95 (C)	300 (T) 156 (C)	0.2142	50 (TT) 49 (TC) 23 (CC)	99 (TT) 102 (TC) 27 (CC)	0.1967	0.9978

Table 10. Family UIMEN050 self report medical history summary. SPAB= spontaneous abortion.

	UIMEN050-A	UIMEN050-U1	UIMEN050-U2	UIMEN050-U3	UIMEN050-U4	UIMEN050-U5	UIMEN050-U6	UIMEN050-U7	UIMEN050-U8
M224T Genotype	CT	CT	TT	CT	CT	TT	TT	CT	TT
Age	55	75	28	46	49	34	64	26	39
Hearing Loss	Yes				Yes				
Tinnitus				Yes					
Vertigo	Yes			Possible					
Aural fullness				Yes		Yes			
Ménière's disease	Definite			Probable	Possible				
Migraine				Yes	Yes		Yes		
Hypertension				Medication	Yes	Yes			
Allergies						Yes			
Crohn's Disease				Son					
Depression	Yes			Yes		Yes			
Edema		Leg							
Other		Diabetes; 2x cervical fusion; triple bypass (smoker)			Cervical fusion				

Table 11. Family UIMEN078 self report medical history summary. SPAB= spontaneous abortion, WNL= within normal limits, Bilat=bilateral, Poss.=Possible, Prob.=Probable.

	UIMEN078-A	UIMEN078-U	UIMEN078-U2	UIMEN078-U3	UIMEN078-U4	UIMEN078-U5	UIMEN078-U6	UIMEN078-U7	UIMEN078-U8	UIMEN078-U9	UIMEN078-U10
M224T Genotype	CT	TT	TT	CT	CT	CC	CT	CT	CT	CT	CT
Age	48	53	22	20	54	46	26	24	21	24	75
Hearing Loss	Yes			Yes	Yes						Bilat
Tinnitus	Yes		Yes	Bilat	Bilat	Bilat	Yes	Yes			Yes
Vertigo	Yes				Yes	Yes	Poss.				Yes
Aural fullness			Yes			Yes					
MD	Poss.		Poss.	Poss.	Prob.	Poss.	Poss.				Prob.
Migraine	Yes		Yes								Poss.
Hypertension	Yes				Poss.	Yes					Yes
Allergies	Yes			Yes	Yes	No				Yes	Pos.
Glasses (before puberty)	Yes		Yes	Yes	Yes		Yes	Yes		Yes	Poss.
Irritable Bowel Syndrome					Yes						Yes
Chronic Rhinitis					Yes	Yes					Poss.
Other	Acoustic neuroma; brain swelling; fluid retention; low potassium & magnesium			Recurrent ear infections & tonsillitis	Gall bladder removed; tubal pregnancy; 2008 Brain MRI WNL	Mitral valve regurgitation					Aortic valve replacement 1992; Hypercholesterolemia; Bilateral peripheral neuropathy; canker sores; Head CT – WNL 2008

Figure 2. Family UIMEN050 pedigree with self report symptoms. *AQP4* M224T genotype listed below individual number.

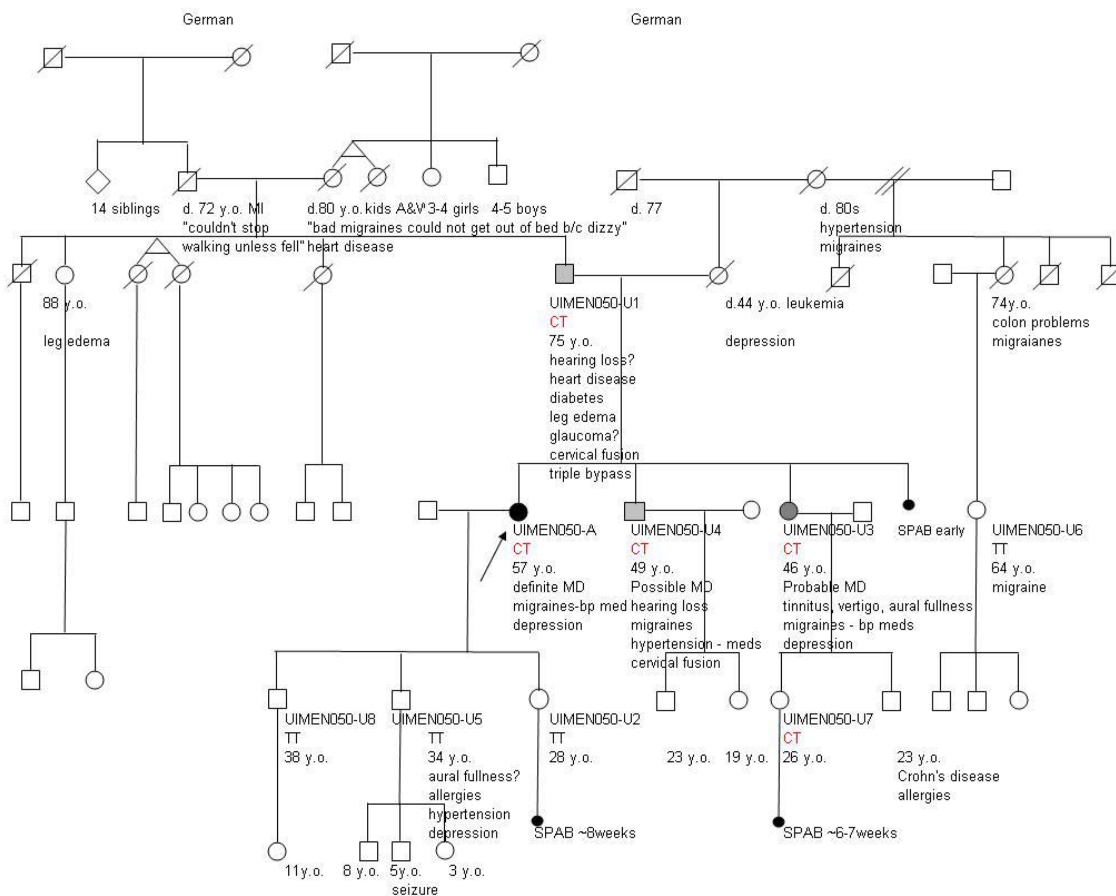


Figure 3. Family UIMEN078 pedigree with self report symptoms. *AQP4* M224T genotype listed below individual number. HL =hearing loss; IBS= Irritable Bowel Syndrome.

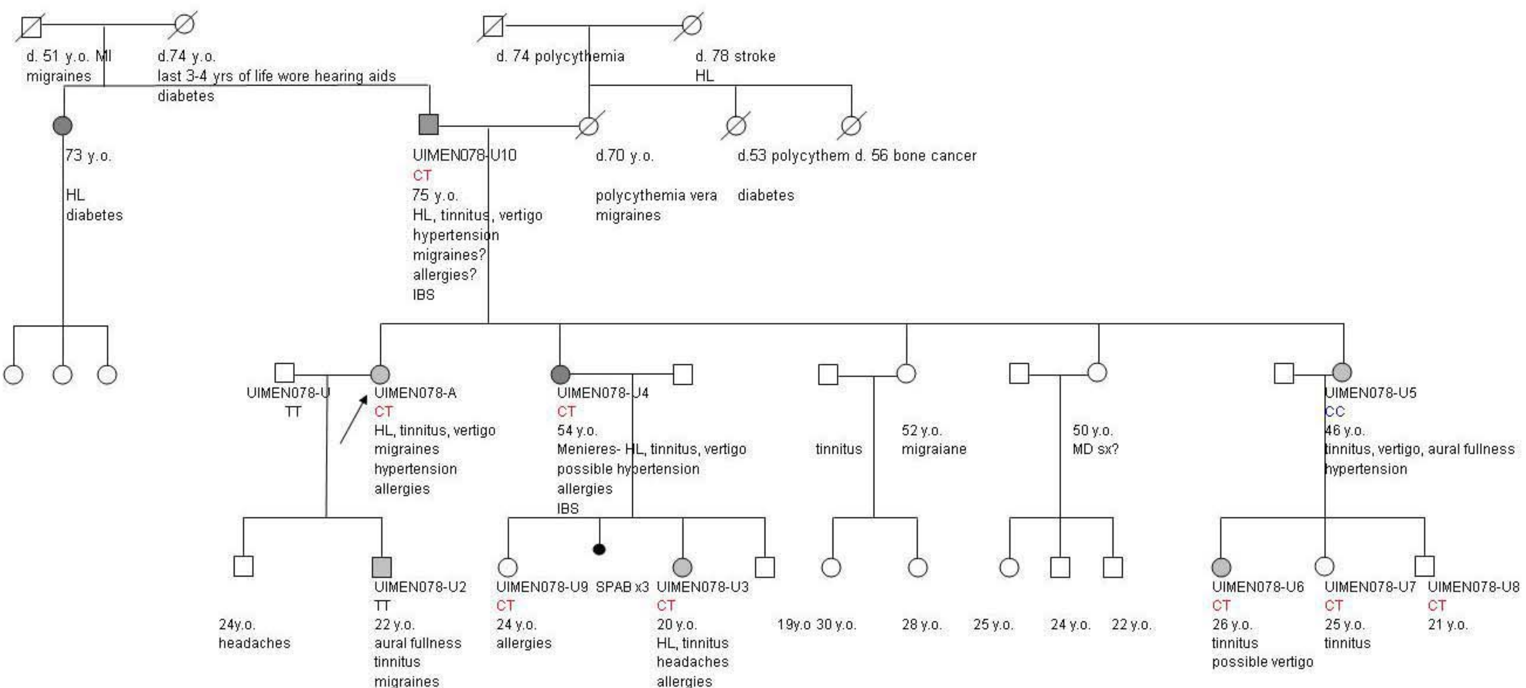


Table 12. Allele and genotype frequencies in *AQP4*.

Exon	SNP (A.A.)	F_1 cases	F_2 cases	F_1 controls	F_2 controls	AA cases	AB cases	BB cases	AA controls	AB controls	BB controls	Controls HWE $\chi2$ p-value	Allele $\chi2$ p-value	Genotype $\chi2$ p-value
5' UTR	rs162008 (NA)	169 (C)	43 (T)	265 (C)	73 (T)	66 (CC)	37 (CT)	3 (TT)	104 (CC)	57 (CT)	8 (TT)	0.9993	0.7130	0.7327
2	240T>G (P65P)	173 (G)	29 (T)	368 (G)	54 (T)	73 (GG)	27 (GT)	1 (TT)	159 (GG)	50 (GT)	2 (TT)	0.7859	0.5913	0.8422
2	405G>A (Q122Q)	198 (G)	4 (A)	416 (G)	6 (A)	97 (GG)	4 (GA)	0 (AA)	205 (GG)	6 (GA)	0 (AA)	0.9786	0.6032	0.8559
3	519G>T (CA) (L160L)	246 (G)	0 (T)	453 (G)	1 (T)	123 (GG)	0 (GT)	0 (TT)	226 (GG)	1 (GT)	0 (TT)	0.9994	0.4613	0.7555
3	rs1839318 (L164L)	241 (C)	5 (T)	448 (C)	6 (T)	118 (CC)	5 (CT)	0 (TT)	221 (CC)	6 (CT)	0 (TT)	0.9801	0.4703	0.6837
4	NEW SNP (M224T)	247 (T)	1 (C)	456 (T)	0 (C)	123 (TT)	1 (TC)	0 (CC)	228 (TT)	0 (TC)	0 (CC)	-	0.1748	0.7567
INT4-5	rs335930 (NA)	145 (A)	39 (C)	287 (A)	81 (C)	56 (AA)	33 (AC)	3 (CC)	113 (AA)	61 (AC)	10 (CC)	0.9486	0.8267	0.6868
INT4-5	rs673629 (NA)	184 (A)	0 (C)	368 (A)	0 (C)	92 (AA)	0 (AC)	0 (CC)	184 (AA)	0 (AC)	0 (CC)	-	-	-
INT4-5	rs507571 (NA)	182 (A)	0 (G)	362 (A)	0 (G)	91 (AA)	0 (AG)	0 (GG)	181 (AA)	0 (AG)	0 (CG)	-	-	-

Table 12-continued

INT4-5	IVS4-67T>C (NA)	197 (T)	51 (C)	368 (T)	86 (C)	80 (TT)	37 (TC)	7 (CC)	150 (TT)	68 (TC)	9 (CC)	0.9674	0.6043	0.7683
5	NEW SNP (T273T)	247 (A)	1 (G)	453 (A)	1 (G)	123 (AA)	1 (AG)	0 (GG)	226 (AA)	1 (AG)	0 (GG)	0.9994	0.7592	0.6629
5	rs3906956 (M278T)	248 (A)	0 (G)	454 (A)	0 (G)	124 (TT)	0 (TC)	0 (CC)	227 (TT)	0 (TC)	0 (CC)	-	-	-
3'UTR	rs9944962 (NA)	244 (A)	0 (T)	456 (A)	0 (T)	122 (AA)	0 (AT)	0 (TT)	228 (AA)	0 (AT)	0 (TT)	-	-	-
3'UTR	rs1058423 (NA)	244 (A)	0 (G)	456 (A)	0 (G)	122 (AA)	0 (AG)	0 (GG)	228 (AA)	0 (AG)	0 (GG)	-	-	-
3'UTR	NEW SNP (NA)	243 (T)	1 (C)	455 (T)	1 (C)	121 (TT)	1 (TC)	0 (CC)	227 (TT)	1 (TC)	0 (CC)	-	0.7693	0.6523
3'UTR	rs9807747 (NA)	244 (A)	0 (G)	456 (A)	0 (G)	122 (AA)	0 (AG)	0 (GG)	228 (TT/AA)	0 (TC/AG)	0 (GG/CC)	-	-	-
3'UTR	rs3763043 (NA)	171 (G)	73 (A)	311 (G)	145 (A)	60 (GG)	51 (AG)	11 (AA)	104 (GG)	103 (AG)	21 (AA)	0.9051	0.6087	0.8088
3'UTR	NEW SNP (NA)	246 (T)	0 (G)	444 (T)	0 (G)	123 (TT)	0 (TG)	0 (GG)	222 (TT)	0 (TG)	0 (GG)	-	-	-
3'UTR	rs335929 (NA)	195 (A)	51 (C)	355 (T)	89 (G)	75 (AA)	45 (AC)	3 (CC)	142 (TT)	71 (TG)	9 (GG)	0.9997	0.8299	0.5475

Table 12-continued.

3'UTR	rs1058424 (NA)	193 (T)	53 (A)	358 (T)	86 (A)	77 (TT)	39 (AT)	7 (AA)	145 (TT)	68 (AT)	9 (AA)	0.9799	0.4950	0.7492
3'UTR	NEW SNP (NA)	246 (C)	0 (T)	443 (C)	1 (T)	123 (CC)	0 (CT)	0 (TT)	221 (CC)	1 (CT)	0 (TT)	-	0.7642	0.1785
3'UTR	NEW SNP (NA)	245 (C)	1 (T)	444 (C)	0 (T)	122 (CC)	1 (CT)	0 (TT)	222 (CC)	0 (CT)	0 (TT)	-	0.7642	0.1785
3'UTR	rs14393 (NA)	174 (C)	72 (A)	305 (C)	139 (A)	62 (CC)	50 (AC)	11 (AA)	103 (CC)	99 (AC)	20 (AA)	0.9266	0.5779	0.7590

Figure 4. Test Cohort *AQP4* Haploview Results. A significant association was not detected between haploblocks and MD in the test cohort.

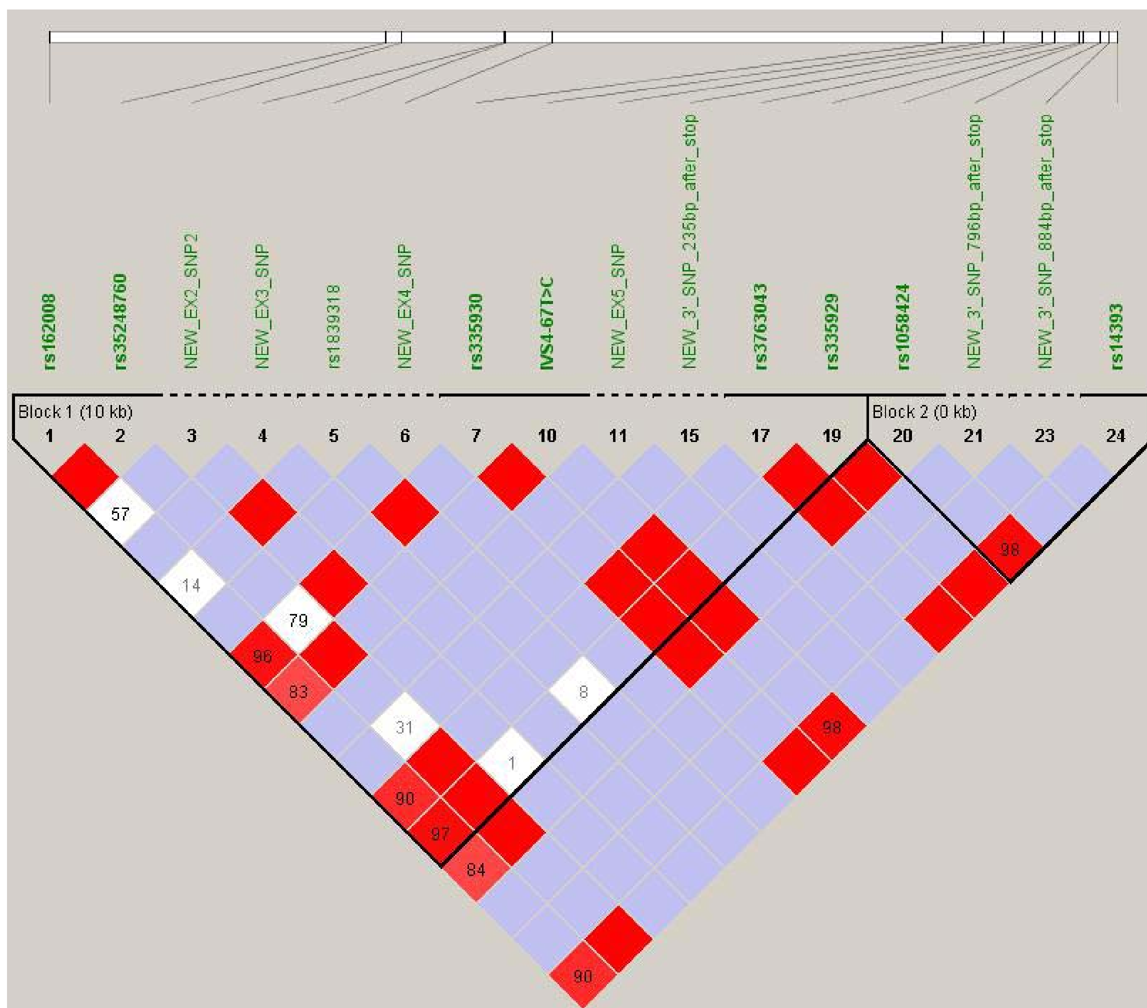


Figure 5. Family UIMEN050 pedigree with *AQP4* STRP haplotypes surrounding the M224T variant.

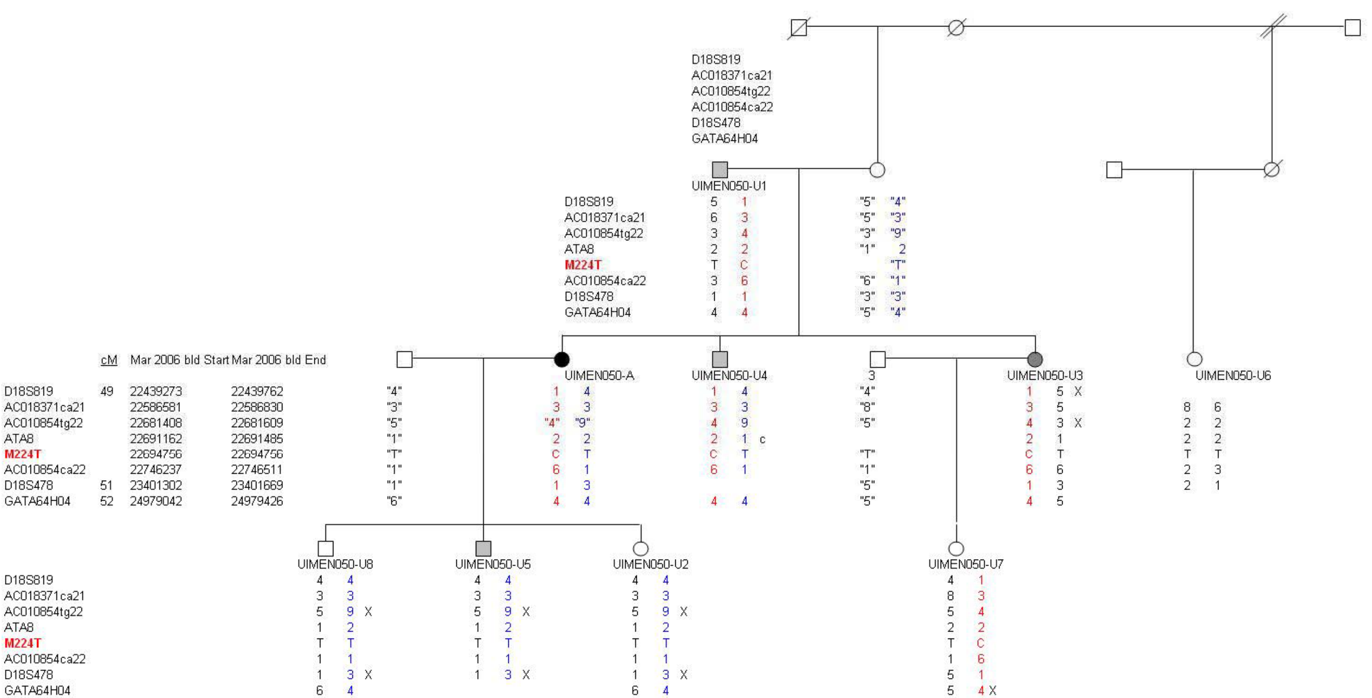
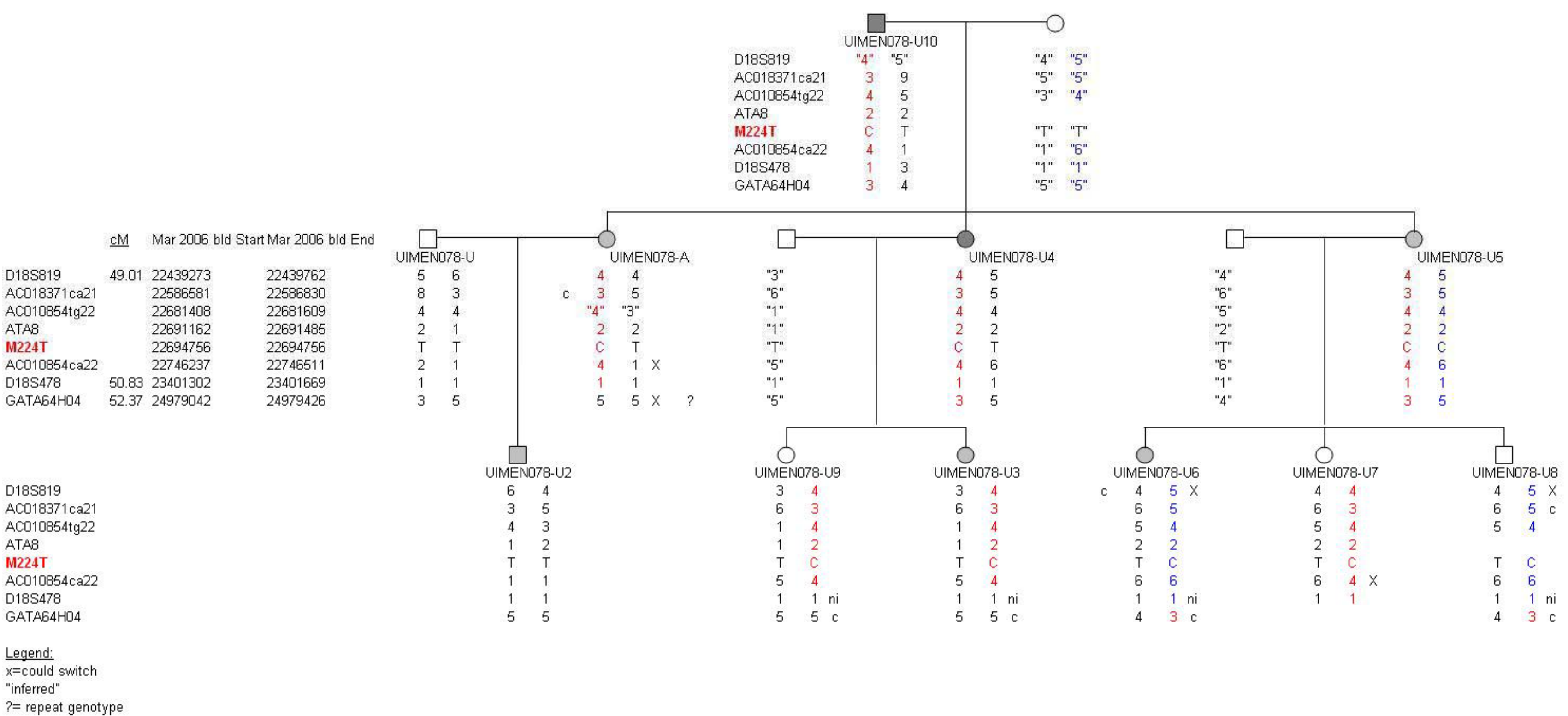


Figure 6. Family UIMEN078 pedigree with *AQP4* STRP haplotypes surrounding the M224T variant.



Discussion

Although none of the SNPs or haploblocks were associated with MD, there were two rare polymorphisms each identified in one patient and none of the controls. The first variant, a non-synonymous SNV in exon 4 (M224T) was found initially in one patient (UIMEN050-A), and later in a second patient (UIMEN078-A) (Table 12). The second variant was a C→T in the 3'UTR of *AQP4*, 235 base pairs after the stop codon. The first patient with M224T has definite MD, and the second patient began developing MD symptoms a year following surgery for an acoustic neuroma. We obtained detailed family and medical histories from these two patients (probands) and their families along with DNA from additional family members. Family members were all screened for M224T in *AQP4* exon 4. The variant segregates with MD symptoms in an autosomal dominant manner in both families. In addition, analysis of STRP markers on chromosome 18 suggests these two families may share a small region of autozygosity by descent surrounding M224T.

Detailed family histories revealed family members with possible and partial MD in both families. Some individuals with the variant do not report symptoms, however it is important to note medical histories were obtained by self report and family members have not been examined by a physician for MD symptoms, with the exception of UIMEN078-U4 who prior to this study was diagnosed with probable MD. Several individuals with the variant are young and may not have developed symptoms yet, and MD is only 60-90% penetrant (Morrison 1995; Frykholm, Larsen et al. 2006). Surprisingly, additional phenotypes were noted including hypertension and migraine suggesting syndromic MD may exist within these two families.

Screening of the HGDP-CEPH Human Genome Diversity Cell Line Panel of controls revealed two of the 1051 controls genotyped (0.19%) have the M224T variant. This panel is a collection of lymphoblastoid cell line DNA from 51 populations around

the world (Cann, de Toma et al. 2002). The controls with the variant are male and of French Basque and Russian descent. However, this SNV was not seen in 424 matched control individuals (848 control chromosomes) indicating this variant is rare in the general population.

M224T is located in exon 4 of *AQP4*, and has a conservation score of 3 on a scale of 1=not conserved, 9=highly conserved, and is conserved among mammals (Figure 7). Among other aquaporins, the threonine is conserved at position 224, and only *AQP4* and *AQP5* have methionine at that position, suggesting the mutation is not random (data not shown; (Khademi 2010)). The rare allele (C allele) is predicted to create c-Ets and STATx transcription factor binding sites (Heinemeyer, Wingender et al. 1998), and also potentially alter the exonic splice enhancer sites, SF2/ASF and Srp40 (http://rulai.cshl.edu/cgi-bin/tools/ESE3/) (Cartegni, Wang et al. 2003; Smith, Zhang et al. 2006).

A review of the literature found M224T decreases water permeability (Sorani, Zador et al. 2008). The variant is located in the extracellular domain of *AQP4*, and at the end of the short pore helix (half-pore helix). Based on the crystal structure of *AQP4*, M224T may change the orientation of the short pore helix which is proposed to be important for water conductance as well as blocking proton leakage. Alternatively, it may also change the dipole of the short pole helix by changing a neutral or hydrophobic residue (Met) to a polar residue (Thr). Or, it may alter the orientation of the loop and thereby alter water conductance (Khademi 2010). Since *AQP4* is expressed throughout the body we anticipate this variant to result in an altered functioning channel, as a non-functioning channel might result in a more severe phenotype. Previous work has demonstrated *Aqp4* expression decreases with age (Christensen, D'Souza et al. 2009). Since multiple aquaporins are expressed in the ear, it may be possible for other aquaporins to compensate for a mutant M224T channel that may not work as well. However, if expression of other aquaporins also decreases with age, the ability to

compensate for the mutant M224T channel may be lost with age resulting in adult onset MD symptoms. Therefore, other aquaporins should be screened in these families as additional variants may contribute to syndromic MD.

The second variant identified in only one MD patient and none of the controls is located in the 3' UTR of *AQP4*, 235 base pairs after the stop codon. We do not yet know the predicted function of this variant in the 3'UTR, however, the rare C allele, is predicted to delete a CdxA transcription factor binding site and create an SRY site, HFH-2 site, and a C/EBPa transcription factor binding site. It is also predicted to delete an SRp55 ESE site (http://fastsnp.ibms.sinica.edu.tw) (Yuan, Chiou et al. 2006). It does not appear to alter an miRNA regulatory site, although it is conserved in mammals (http://genome.ucsc.edu/) (Kent, Sugnet et al. 2002).

Prior to this study families segregating MD and migraine and individuals with MD and glaucoma had been reported suggesting a syndromic form of the disease may exist (Brown 1941; Brown 1949; Godtfredsen 1949; McGrath 1952; Morrison and Johnson 2002; Oliveira, Ferrari et al. 2002; Ruckenstein, Prasthoffer et al. 2002; Boyev 2005) (Appendix D). However, a disease causing gene had not been reported. In our own experience, 25/101 (24.8%) of MD patients reported a personal of family history of migraine, and 13/101 (12.9%) of MD patients reported a personal of family history of glaucoma when questioned (unpublished results).

Both families in the current study report migraine, hypertension, allergies, Irritable Bowel Syndrome or Crohn's disease, edema and heart disease (Table 13). In addition, the patient with the 3'UTR variant (UIMEN024-A) has a personal history of ulcerative proctitis. Interestingly, previous studies have shown AQP4 has decreased expression in mice and patients with Crohn's colitis and infectious colitis, and altered water and electrolyte transport is associated with colitis (Hardin, Wallace et al. 2004). AQP4 is expressed throughout the body including the stomach, small intestine, and large intestine, as well as in the lung, eye, and kidney (Matsuzaki, Tajika et al. 2004).

AQP4 is highly expressed in the brain and *Aqp4* deficient mice models are protected and have reduced cytotoxic cellular edema, whereas *Aqp4* deficient mouse models with vasogenic extracellular edema have worsened edema suggesting a role for Aqp4 in cerebral water transport (Bloch and Manley 2007). A candidate gene association study for migraine and *AQP4* did not reveal an association between migraine and *AQP4*, however only 4 variants were genotyped so rare variants may have been missed (Rubino, Rainero et al. 2009). In the near future it will be necessary to carefully define the phenotypes in these two families through physical exam, collection of additional medical records, and audiometric testing. It may be helpful to perform an epidemiologic study to investigate if migraine, hypertension, allergies, intestinal diseases, edema (brain or extremities), and heart disease are found at a higher rate in individuals with MD or if these additional phenotypes are only seen in a subset of families with syndromic MD. Additional MD patients should also be screened for rare variants in *AQP4* as we have only screened 124 patients for the entire gene in the current study.

In summary, although common variants in *AQP4* were not associated with the MD in this study, we did identify two rare variants in persons with MD. We hypothesize that these variants impact endolymph homeostasis thereby influencing the development of MD. This study is significant because it is the first gene associated with MD. This study is also significant because it is the first association of aquaporins with human hearing loss, in particular *AQP4*. This study may help elucidate factors involved in the regulation of endolymph and the formation of endolymphatic hydrops. In addition, this study is significant as it is the first report of a gene for syndromic MD.

Figure 7. *AQP4* M224T Conservation from UCSC Genome Browser.

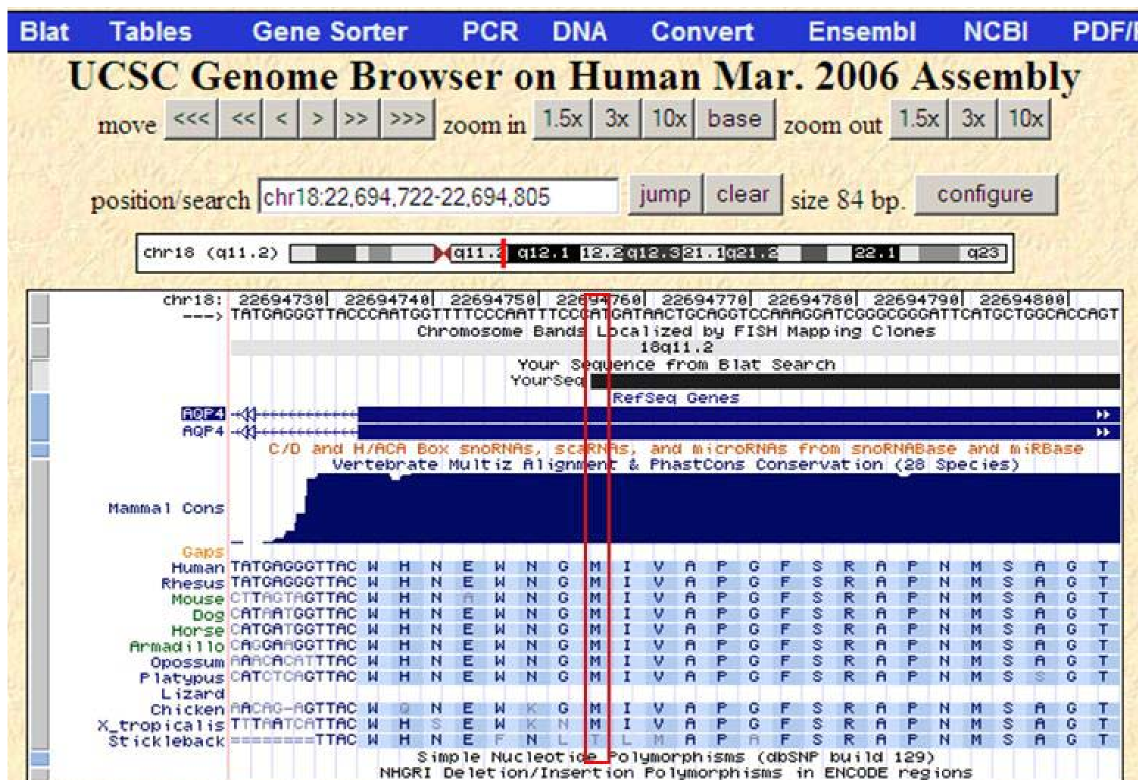


Table 13. Shared phenotypes among families with *AQP4* variants. A detailed family history has not yet been obtained for UIMEN024-A.

	UIMEN050	UIMEN078	UIMEN024
Migraine	x	x	
Hypertension	x	x	
Allergies	x	x	
Irritable Bowel Syndrome; Crohn's disease; Ulcerative proctitis	x	x	x
Chronic Rhinitis		x	
Depression	x		
Edema	x	x	
Heart disease	x	x	
Acoustic neuroma		x	
Brain edema		x	

CHAPTER IV

MAPPING OF A NOVEL MENIERE'S DISEASE LOCUS TO CHROMOSOME 1

Abstract

Ménière's disease (MD) is a complex disorder of unknown etiology characterized by the symptom triad of vertigo, sensorineural hearing loss and tinnitus. Most cases of MD are sporadic and only occasional families are identified with multiple affected persons, making classical linkage analysis difficult. We identified a Chilean family segregating autosomal dominant MD over three generations and completed a genome-wide linkage scan using the Affymetrix GeneChip® Mapping 50K array. Multi-point parametric linkage analysis assuming dominant inheritance identified probable linkage to chromosome 1q32.1-q32.3 with a maximum lod score of 2.36. The candidate gene interval determined by haplotype reconstruction spanned 8.3 Mb (201.71- 210.29cM) and included 117 known or hypothetical genes. Exon capture followed by pyrosequencing was performed for all known and predicted exons in the interval on DNA from a parent-child trio within the family. Filtering of variants identified two changes that segregate with the disease phenotype, one each in *PCTK3* and *SLC45A3*. A sporadic MD population and matched controls were screened and rare and common variants were found to be associated with both genes. Since little is known about MD and its initiating factors, the identification of the genetic cause(s) of MD in this family may clarify aspects of disease pathogenesis in both familial and sporadic MD and lead to better treatment strategies in affected patients. Chapter 4 is in preparation for publication.

Introduction

Ménière's disease (MD) is a complex disorder of the inner ear characterized by the symptom triad of hearing loss, tinnitus, and vertigo (1995). Although it was first

described by Prosper Ménière almost 150 years ago (Meniere 1861), surprisingly little is known about its pathogenesis and recognizing MD can be a diagnostic challenge. For that reason, the American Academy of Otolaryngology-Head and Neck Surgery (AAO-HNS) categorizes MD as 'definite', 'certain', 'probable' and 'possible'. 'Definite' MD is an exclusionary diagnosis that requires the documentation of two or more attacks of spontaneous vertigo each greater than 20 minutes duration, hearing loss, documented on at least one occasion, and tinnitus or aural fullness in the affected ear. 'Certain' MD can only be diagnosed on autopsy and includes the above criteria plus histopathologic confirmation of endolymphatic hydrops (EH). 'Probable' MD implies one attack of vertigo with documented hearing loss and tinnitus or aural fullness in the affected ear. 'Possible' MD should be considered if MD-type vertigo occurs without hearing loss or if fluctuating sensorineural hearing loss without vertigo is documented (1995).

Symptoms typically present in the 4th decade without an identifiable inciting event. Vertigo experienced by patients can be extremely debilitating. Following onset of disease, patients may experience problems for days or months, or be symptom-free for years. About 70% of patients benefit from symptomatic treatment, which includes dietary restrictions, steroids, diuretic therapy and vestibular rehabilitation exercises (Sajjadi 2002; Thorp, Shehab et al. 2003; Kim, Wiet et al. 2005; Thirlwall and Kundu 2006).

The incidence of MD in Caucasians is 1-2 per 1000 (Harrison 1968; Morrison 1995). Most cases are sporadic, although occasional families segregating MD are reported (Martini 1982; Oliveira and Braga 1992; Morrison 1995). To date only one familial MD locus has been mapped (12p12); the causative gene at this locus has not been identified (Klar, Frykholm et al. 2006). In this paper we describe a Chilean family segregating autosomal dominant MD with variable penetrance over three generations. The family does not report consanguinity. Using a genome-wide mapping strategy, we localized the MD gene in this family to chromosome 1q32.1-1q32.3 and screened all

coding exons of known and hypothetical genes in the linked interval in a parent-child trio using targeted capture and pyrosequencing.

Materials and Methods

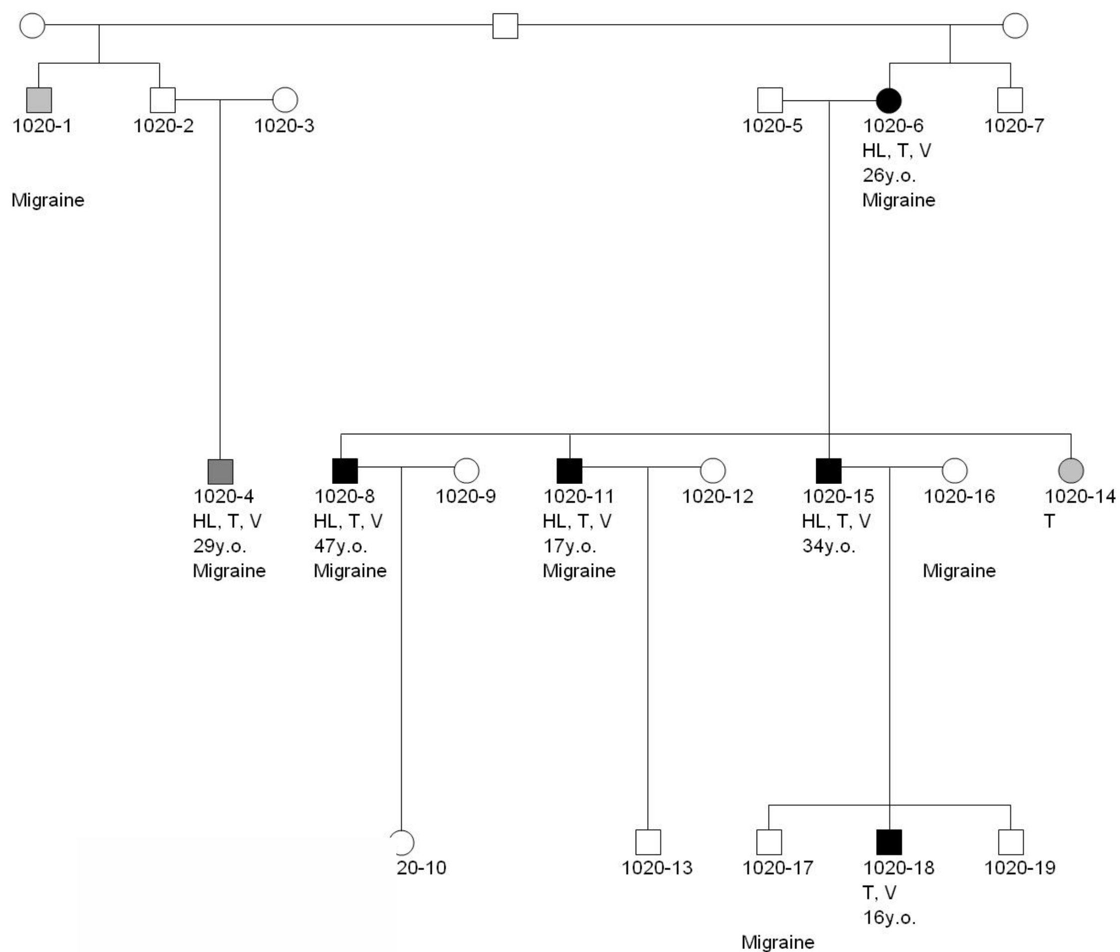
Patients

In participating, consenting persons, AAO-HNS published criteria were used to diagnose MD (Figure 8) (1995). Genomic DNA (gDNA) was extracted from peripheral blood or saliva with a Gentra Puregene or Oragene kits, respectively, according to manufacturer's instructions (QIAGEN, Valencia, CA; http://www1.qiagen.com/, Oragene, DNA Genotek, Ottawa, Ontario, Canada; http://www.dnagenotek.com). DNA was quantitated in triplicate using a ND-1000 Spectrophotometer V3.5 (Wilmington, DE, USA). All procedures were approved by the Institutional Review Board at the University of Iowa.

Affymetrix GeneChip® Mapping 50K SNP Genotyping

Genomic DNA was amplified, fragmented and precipitated as described (Matsuzaki, Dong et al. 2004) (Appendix C). SNP genotyping was completed using Affymetrix GeneChip® Human Mapping 50K Xba 240 Arrays according to manufacturer's instructions (Affymetrix, Santa Clara, CA) at the Translational Genomics Research Institute (TGen, Phoenix, Arizona). Arrays were processed for the single base extension reaction, stained and scanned by a GeneChip Scanner 3000 7G (Affymetrix, Santa Clara, CA). Normalized bead intensity data obtained for each sample were loaded into the GeneChip Operating Software (GCOS) and GeneChip DNA Analysis Software (GDAS). (DNA from every family member except 1020-1 was run on a separate array.) The Affymetrix GeneChip® Human Mapping 50K Array Xba 240 uses >50,000 on a single chip with an average marker distance of 26 kb.

Figure 8. Family 1020 pedigree. HL= Hearing Loss; T=Tinnitus; V= Vertigo. Age listed is the age at diagnosis of MD. Black circle=affected female; black square=affected male; light gray circle= female with partial disease; light gray square=male with partial disease; dark gray square= male, possible phenocopy.



Linkage Analysis

The Affymetrix BRLMM Analysis Tool was used to determine allele calls. Data were analyzed with DNA-Chip Analyzer software (dChip) (www.dchip.org) using multi-point parametric linkage analysis and assuming autosomal dominant inheritance. Regions with lod scores greater than 1.0 were further analyzed. X-linked inheritance was excluded as both males and females were affected with Ménière's disease in this family.

Short Tandem Repeat Polymorphic (STRP) Marker Confirmation

STRP markers were selected for each candidate interval with a lod >1.0 and amplified for the entire family to reconstruct haplotypes, as described in Appendix C. GeneMapper v4.0 was used to assign alleles. Haplotypes were manually reconstructed.

NimbleGen Targeted Capture

The linked interval on chromosome 1q32.1-1q32.3 spanned chr1: 203523961-211830820 (8,306 kb). Roche NimbleGen designed and manufactured a custom Sequence Capture 385K Human Array covering all known and hypothetical exons in this region (753 exons in 117 genes, 0.518Mb) with 385,000 non-unique probes (Roche NimbleGen, Madison, WI, USA). Exons less than 50 base pairs (bp) that could not be merged with a neighboring exon were not included in the design. All exons were padded with 30bp on the 5' end and 15bp on the 3' end to cover splice variants. Targets were brought up to a minimum size of 500bp. Targets were verified with SignalMap version 1.9 software (Roche NimbleGen, Madison, WI, USA). The array design predicted 29-fold coverage for at least 50% of the bases, and 12-fold coverage for at least 90% of the requested bases.

Four DNA samples were captured separately – two from the proband (1020-15) obtained one year apart, one from his unaffected spouse (1020-16) and one from his

affected son (1020-18) to allow for biological replicates of the proband as well as segregation analysis within the family. Sequence capture and 454 pyrosequencing was performed at the 454 Life Sciences Service Center (454 Sequencing Center, 1 Commercial Street, Branford, CT 06405) using their modified protocol with 454 adaptors. Each captured DNA sample was placed in a gasket on one 454 GS FLX titanium chemistry run, expecting 30-55Mb sequence data per gasket.

Sequence Data Analysis

Linker sequences were removed *in silico* and sequences compared to the Human Genome version NCBI Build 36.1. Sequence reads which did not map back uniquely to the Human Genome version NCBI Build 36.1 were discarded. Following assembly of the sequencing reads and comparison with the reference sequence, discrepancies between the patient samples and the Human Genome version NCBI Build 36.1 were identified with GS Amplicon Variant Analyzer software (454 Life Sciences, Branford, CT, USA).

Filtering Strategy

Variants for each sample from the high confidence differences (HCDiffs) variant list were prioritized by the following criteria: map to region, coding, absence from dbSNP128 not present in the unaffected sample (1020-16), non-synonymous or synonymous. The same filtering strategy was performed for the all variants (AllDiffs) list for each sample as well as Sanger validation, and used for the remaining analysis. Variants which passed the AllDiffs filtering criteria were analyzed for segregation with MD in the family. Sanger sequencing was used to verify variants. A 36bp deletion was visualized by running the PCR product of *SLC45A3* exon3-2 amplimer on a 2% agarose gel run at 80 volts for 5 hours, and deletion boundaries confirmed by Sanger sequencing (Figure 9).

Autozygosity-By-Descent Analysis

Di-, tri-, and tetra- nucleotide repeats were identified in the genomic region spanning *SLC45A3*. Primers were designed using Primer 3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) and amplified for all family members as described in Appendix C. GeneMapper v4.0 was used to assign alleles. Haplotypes were reconstructed manually to analyze individuals 1020-5 and 1020-6 for autozygosity.

cDNA Expression Analysis

Primers were designed to cover exon-intron boundaries of the last two exons of candidate genes with Primer 3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). Primers to GAPDH were used to as a positive control. P6 mouse cochlea cDNA was amplified under standard conditions. PCR products were resolved on a 2% agarose gel and Sanger sequenced for confirmation.

Rare and Common Variants Study Design

Subjects

A population of 124 Caucasian patients diagnosed with definite MD as well as 124 controls matched for gender, age, and ethnicity were Sanger sequenced for the coding regions of *PCTK3* and *SLC45A3*. The patient and control cohorts were each comprised of 68 females and 56 males (1:1.2 female: male ration) with a mean age of 56.2 years or 55.9 years for patients and controls, respectively. Population substructure was ruled out after genotyping the *LCT* promoter SNP rs4988235, with a genotype p-value of 0.6083 (Table 14).

Candidate Genes Screen

Primers for all coding exons and intronic boundaries were designed with Primer 3 as described in previous chapters. All exons were amplified in all patients and all controls and sequencing performed as described in prior chapters. All sequences were

read and rare and common variants noted. Haploblocks were obtained from HapMap (http://hapmap.ncbi.nlm.nih.gov/) (2003). By sequencing the entire gene(s) if 10 rare variants with a frequency of 0.001 are present in a gene, sequencing 100 individuals has a 90% chance of identifying one of the 10 variants (Li and Leal 2009).

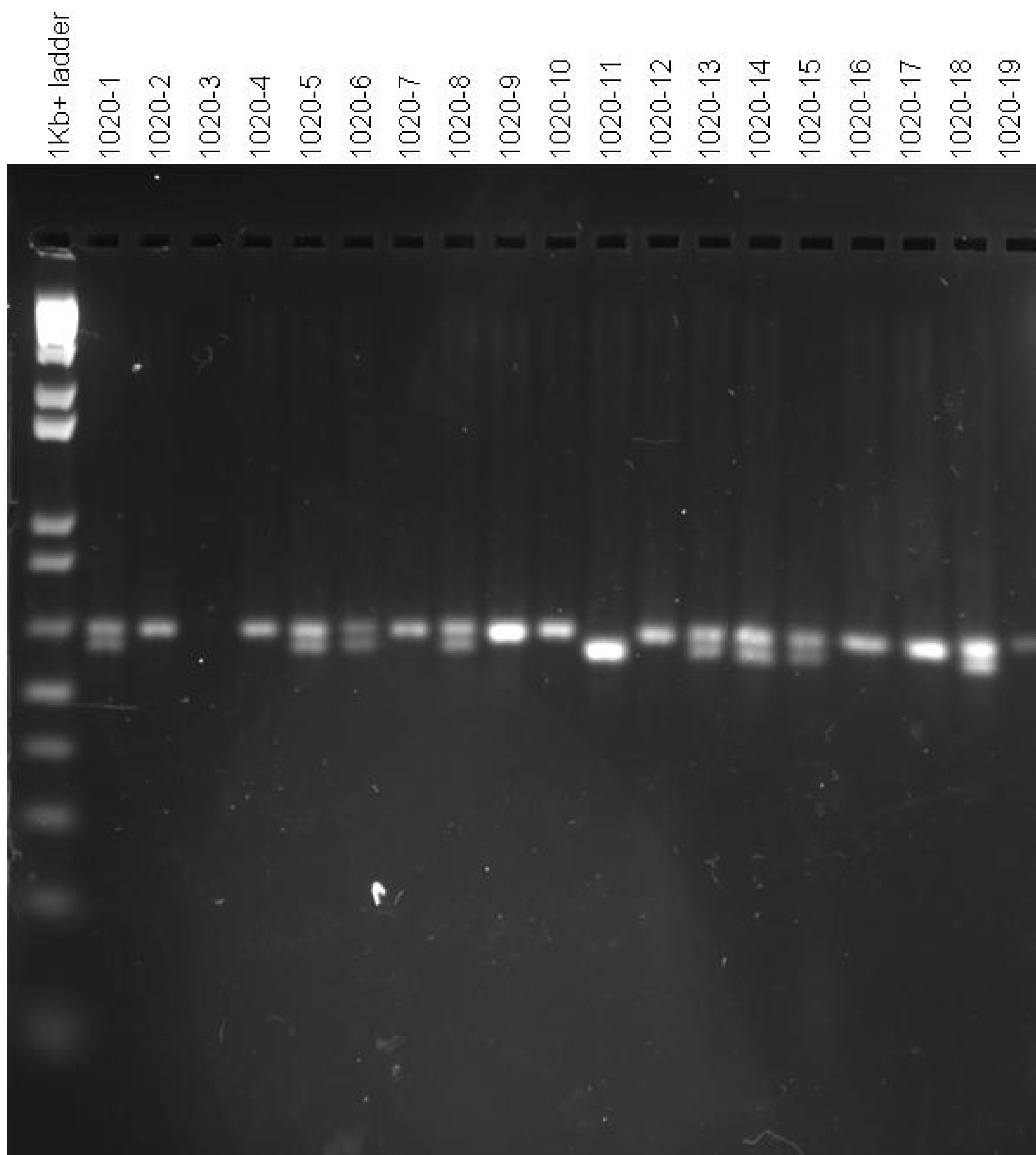
Table 14. Allele and genotype frequencies for *LCT* promoter SNP rs4988235 (Campbell, Ogburn et al. 2005).

A. Genotype and allele frequencies for rs4988235						
	TT	TC	CC	T	C	Total
MD	50 (41.0%)	49 (40.0%)	23 (19.0%)	149 (61.1%)	95 (38.9%)	122
CTRLS	50 (40.3%)	57 (46.0%)	17 (13.7%)	157 (63.3%)	91 (36.7%)	124

B. Iowa MD and Controls *LCT* rs4988235 Results						
Alleles (MD)	Alleles (CTRLS)	χ^2 p-value	Genotypes (MD)	Genotypes (CTRLS)	χ^2 p-value	*HWE p-value (CTRLS)
149 (T) 95 (C)	157 (T) 91 (C)	0.4753	50 (TT) 49 (TC) 23 (CC)	50 (TT) 57 (TC) 17 (CC)	0.6083	0.9965

*HWE = Hardy-Weinberg equilibrium

Figure 9. *SLC45A3* 36 bp deletion. A 36bp deletion was visualized by running the PCR product of *SLC45A3* exon3-2 amplimer on a 2% agarose gel run at 80 volts for 5 hours.



Results

Patients

Five persons in this family had definite MD (1020-6, 1020-8, 1020-11, 1020-18) and one person had possible MD (1020-14). We considered five persons to be unaffected (1020-5, 1020-7, 1020-9, 1020-12, 1020-16), and four persons to be too young to classify (1020-10, 1020-13, 1020-17, 1020-19). Saliva only was obtained from 1020-1 who had possible MD (Figure 8, Table 15).

Linkage and Haplotype Analyses

The range of genotyping calls on the Affymetrix GeneChip® Mapping 50K Array was 90.6-99.8% with a mean call rate of 97.9%. Multi-point parametric linkage analysis assuming dominant inheritance identified probable linkage to chromosome 1q32.1-q32.3 with a maximum lod score of 2.36 (rs1269860 (SNP_A-1756763)) (Figure 10). A second linkage peak was noted on chromosome 17p12 with a maximum lod score of 1.66 (rs2521892 (SNP_A-1719505)). The only other linkage regions with a lod score >1.0 were two additional regions on chromosome 17 (Figure 11). (1020-1 was not included in the linkage analysis as there was not enough DNA to run on the microarray.)

Haplotype reconstruction of the chromosome 17 region showed that STRP markers did not segregate with MD thereby excluding this interval. The chromosome 1q32.1-q32.3 region as defined by haplotype reconstruction using STRPs and SNPs, spanned 8.3 Mb (201.71- 210.29cM) (Figure 10). This interval includes 79 known genes, 8 open reading frames and 30 hypothetical proteins; 22 genes had known expression in the mouse inner ear.

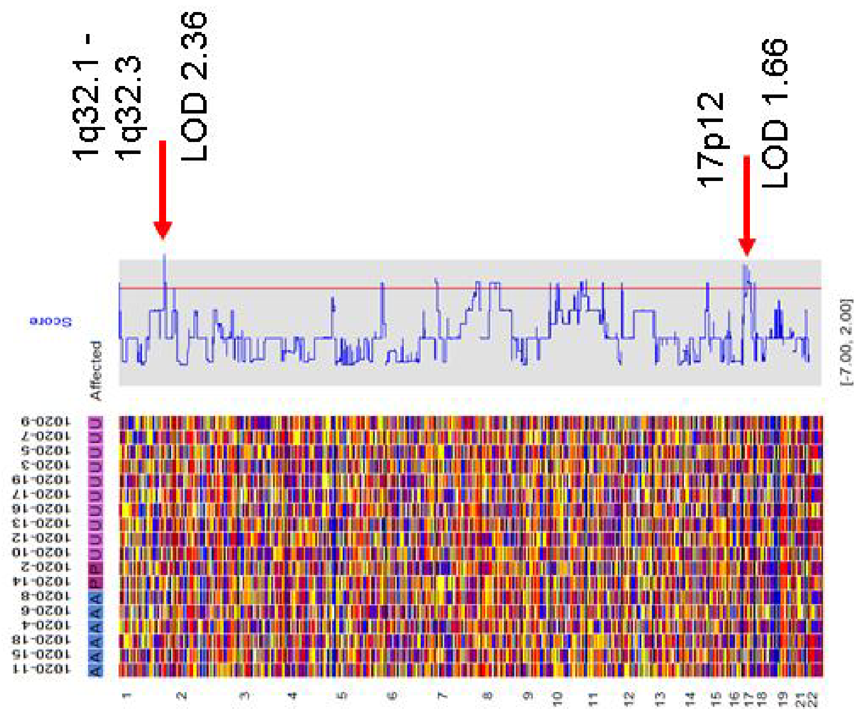
Targeted Sequence Capture and Deep Sequencing

The custom NimbleGen Target Array included all known and hypothetical exons in the chromosome 1q32.1 – 1q32.3 linkage region resulting in a total of 1959 targets covering 1,559 kb. Two DNA samples from the proband (1020-15a and 1020-15b), one from his unaffected spouse (1020-16), and one from his affected son (1020-18) were captured separately. Overall, each sample had between 137,479-218,824 high-quality reads and 50,207,144 – 80,449,071 high-quality bases, with a mode read length of ~500bp (Table 16). About 70% of reads mapped inside the target regions indicating successful enrichment with an average target base coverage of 97.4% and an average capture base coverage of 99.25%. For each sample, 1400-1700 high confidence variants were identified with ~90% annotated by dbSNP128 (Table 17). Each sample had between 1909-2423 total variants, with ~74% annotated by dbSNP128 (AllDiffs) (Table 18).

Table 15. Participants' symptoms. Legend, T= Tinnitus, HL = Hearing Loss, V= Vertigo

Individual	Male/ Female	Affected Status	Age at diagnosis of MD	History of Migraine? Yes/No
MEN1020-1	M	Apparent symptoms	> 40	Y
MEN1020-2	M	Unaffected	NA	N
MEN1020-3	F	Unaffected	NA	N
MEN1020-4	M	T, HL, V	29	Y
MEN1020-5	M	Unaffected	NA	N
MEN1020-6	F	T, HL, V	26	Y
MEN1020-7	M	Unaffected	NA	N
MEN1020-8	M	T, HL, V	47	Y
MEN1020-9	F	Unaffected	NA	N
MEN1020-10	F	Unaffected	NA	N
MEN1020-11	M	T, HL, V	17	Y
MEN1020-12	F	Unaffected	NA	N
MEN1020-13	M	Unaffected	NA	N
MEN1020-14	F	T	30	N
MEN1020-15	M	T, HL, V	34	N
MEN1020-16	F	Unaffected	NA	Y
MEN1020-17	M	Unaffected	NA	Y
MEN1020-18	M	T, V	16	N
MEN1020-19	M	Unaffected	NA	N

Figure 10. STRP marker confirmation of the candidate interval on 1q32.1-1q32.3. Left: Family segregating Ménière's disease. The proband (red arrow), 1020-15, lives in the United States with his wife and children. The extended family is in Chile. 1020-2,-3 and -4 were not used in the first-pass linkage analysis. Black circle= affected female; black square=affected male; light gray circle=partial disease female; light gray square=partial disease male; dark gray square=possible phenocopy. Disease haplotype indicated in red; horizontal lines indicate interval boundaries. Right: Genome-wide linkage results. The vertical red line represents LOD=0; the right margin of the grey box represents LOD=2. All subsequent studies we have done on this family are based on the largest mapped interval on chromosome 1q, which excludes the genotypic data provided by the right branch of the family (1020-1,-2,-3 and -4) based on the possibility that 1020-4 is a phenocopy. (ni=non-informative).


1q32.1 -
1q32.3
LOD 2.36
17p12
LOD 1.66
Score
Affected
[-7.00, 2.00]

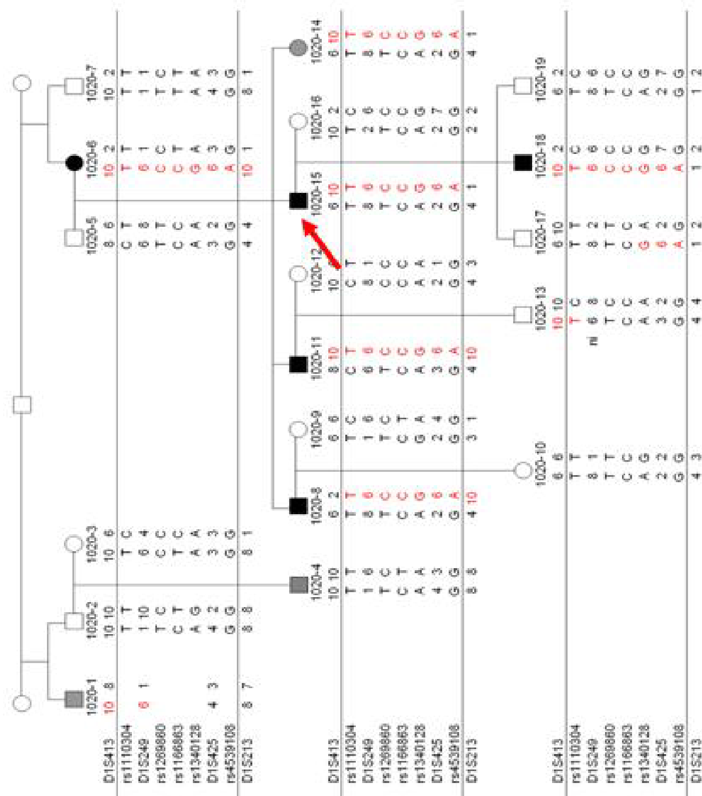


Figure 11. dChip output of chromosome 17 linkage intervals. The region with the second highest overall lod score was on 17p12, and spanned chr17:12656242-13038215 (~382kb). The next two lod scores >1.0 on 17q and the combined interval spanned chr17:29901059-51576678 (~21.7kb).

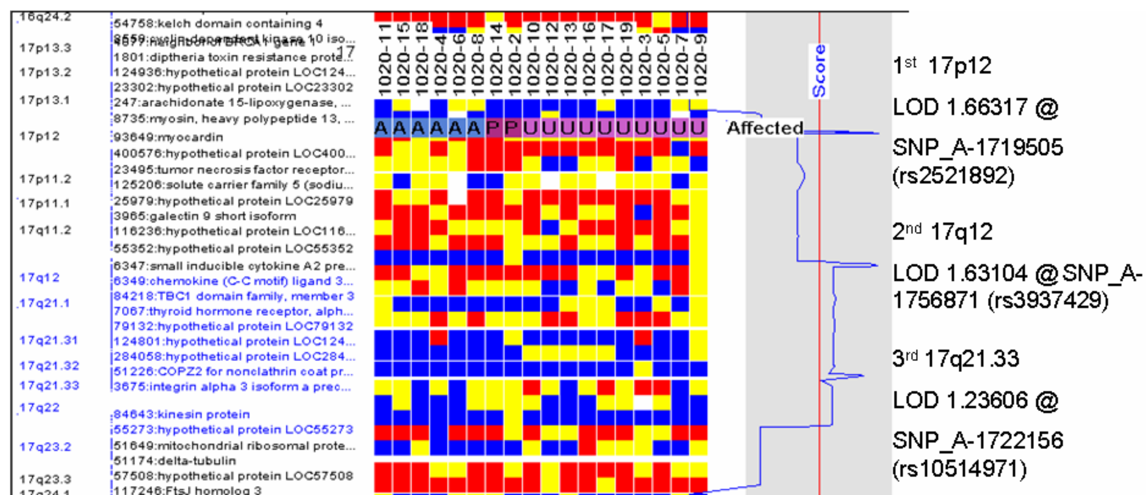


Table 16. Sequence Results Summary

Sample	1020-15a	1020-15b	1020-16	1020-18	Mean
HQ Reads	208,458	137,479	218,824	215,099	194,965
HQ Bases	77,450,924	50,207,144	80,449,071	81,200,961	72,327,025
Ave Read Length	372	365	368	378	371
Mode Read Length	491	485	499	493	492
Percent Mapped Bases	99.66%	99.62%	99.66%	99.73%	99.67%
Percent Mapped Reads	99.22%	99.15%	99.23%	99.38%	99.23%
Reads Uniquely Mapped	90.8%	90.7%	89.8%	90.6%	90.50%
Unique Reads in Region	73.48%	78.10%	71.94%	74.81%	74.58%
Target Base Coverage	97.4%	97.0%	97.6%	97.6%	97.4%
Capture Base Coverage	99.2%	98.9%	99.5%	99.4%	99.3%
Weighted Mean Coverage (Min-Max)	28.1x (1-98x)	19.3x (1-337x)	28.2x (1x-91x)	29.6x (1x-137x)	26.3x

Table 17. High Confidence Variant Results. Variants with >3x depth and 20% frequency.

Sample	1020-15a	1020-15b	1020-16	1020-18	Mean
Total Variants	1628	1416	1756	1737	1634
Novel Variants synonymous nonsynonymous	121 (7.4%) 118 (97.5%) 3 (2.5%)	87 (6.1%) 85 (97.7%) 2 (2.3%)	170 (9.7%) 167 (98.2%) 3 (1.8%)	157 (9.0%) 154 (98.1%) 3 (1.9%)	134 (8.2%) 131 (97.8%) 3 (2.2%)
Known Variants synonymous nonsynonymous	1507 (92.6%) 1478 (98.1%) 29 (1.9%)	1329 (93.9%) 1302 (98.0%) 27 (2.0%)	1586 (90.3%) 1546 (97.5%) 40 (2.5%)	1580 (91.0%) 1544 (97.7%) 36 (2.3%)	1501 1468 (97.8%) 33 (2.2%)

Table 18. All Variants Results

Sample	1020-15a	1020-15b	1020-16	1020-18	Mean
Number of variants	2294	1909	2423	2398	2256
Coding variants	130	102	128	130	122.5
Nonsynonymous variants	67	51	70	67	63.75
Known nonsynonymous variants	39	30	43	39	37.75
Novel nonsynonymous variants	28 (1.2%)	21 (1.1%)	27 (1.1%)	28 (1.2%)	26 (1.2%)
Synonymous variants	63	51	58	63	58.75
Known synonymous variants	51	44	49	51	48.75
Novel synonymous variants	12 (0.5%)	7 (0.4%)	9 (0.4%)	12 (0.5%)	10 (0.4%)

Variant Identification

There were zero novel non-synonymous variants found only in the 3 affected samples and not in the unaffected sample from the HCDiffs list. Forty-five novel non-synonymous variants in 32 genes passed the AllDiffs-filtering criteria: two variants were found in all three affected samples; 25 variants were found in two affected samples; and 18 variants were found in only one affected sample (Figure 12, Table 19). In addition, there were 18 novel synonymous variants in 17 genes: one variant was found in all three affected samples; 11 variants were found in two affected samples; and 6 variants were found in only one affected sample (Table 20). In total three variants were found in all three affected samples but not in the unaffected sample (Figure 13).

Sanger sequencing validated three of the 45 non-synonymous variants, one of which – *SLC45A3* A222_S233del – segregated with the disease in the family (Table 19, Figure 14). The 36 bp deletion was confirmed by visualization on agarose gel, and boundaries validated by Sanger sequencing (Figure 9). Interestingly, individual 1020-11 is homozygous and both parents are heterozygous for *SLC45A3* A222_S233del. STRP markers tightly linked to the deletion showed that 1020-5 and 1020-6 share a common haplotype, consistent with autozygosity by descent (Figure 15). Although originally reported as novel, *SLC45A3* A222_S233del has now been assigned two rs numbers in dbSNP130, rs72434280 and rs71152447.

Of the 18 novel synonymous variants, one was validated by Sanger sequencing and segregated with disease in the family- *PCTK3* L436L (Table 20, Figure 14). The predicted variant in *CR1* is in a homologous region that cannot be uniquely sequenced by Sanger sequencing and has therefore not been validated. The predicted variant in LOC642587 is a tri-nucleotide repeat, and fluorescently labeled STRP markers are being optimized to validate this variant.

Cochlear and Endolymphatic Sac Expression

Expression of *Gapdh* (positive control), *Pctk3*, and *Slc45a3* cDNA was detected (Figure 16) and confirmed by sequence analysis in the P6 mouse cochlea. Primers are being optimized for human *PCTK3* and *SLC45A3* cDNA expression in human fibroblasts.

Figure 12. AllDiffs Variant Filtering Strategy.

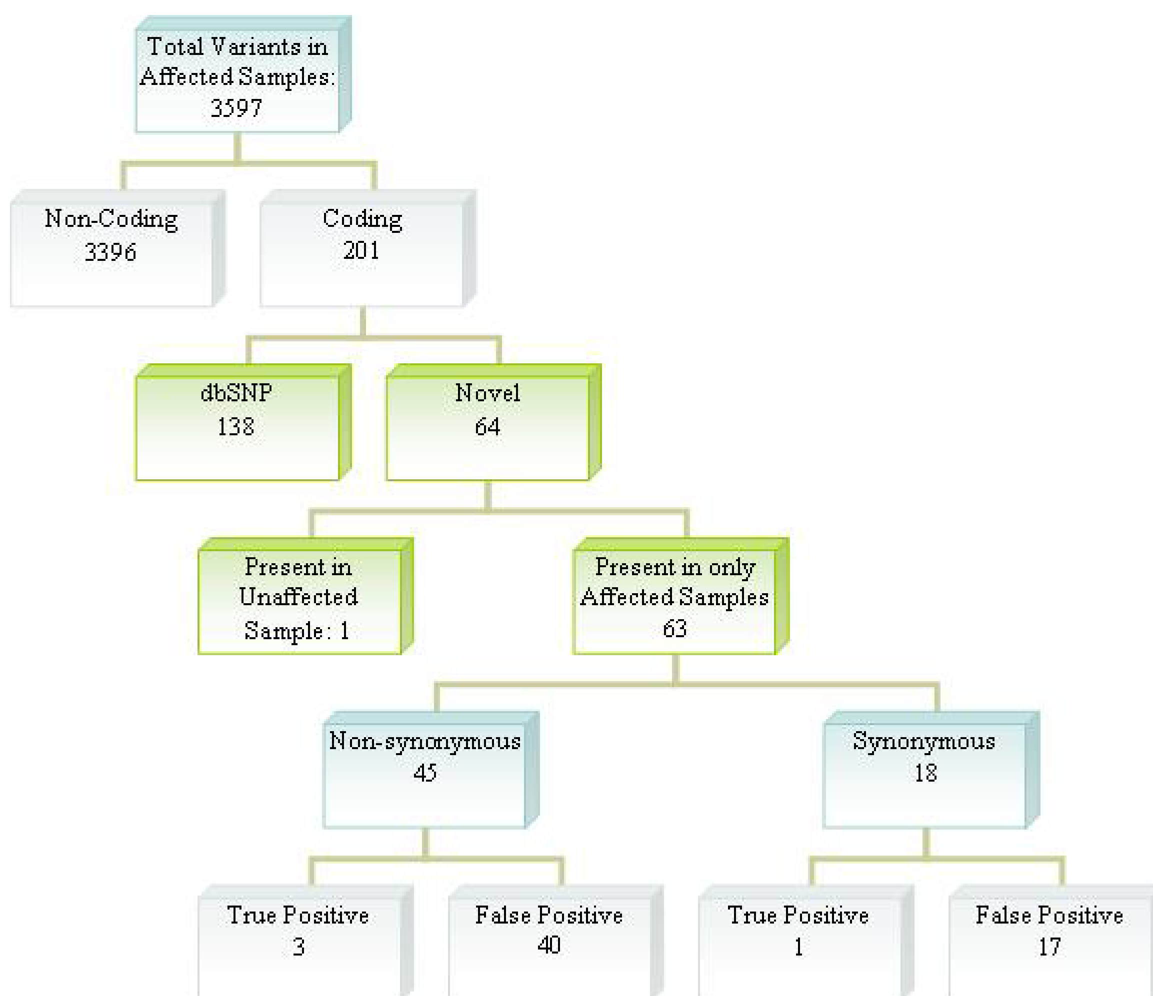


Table 19. 45 unique, novel, chromosome 1 nonsynonymous variants. All variants are within linkage breakpoints. The variants in *CR1* and LOC642587 have not been validated by Sanger sequencing due to inability to design unique primers, and a tri-nucleotide repeat, respectively.

Sample												
1020-15a	1020-15b	1020-18	Location	Ref Nuc	Var Nuc	Total Depth	Var Freq	Ref AA	Var AA	Gene	Sanger Validated	Familial segregation
x	x	x	chr1:203898843 - 203898877	CGACAGCCCTTCTGC TGGCTCGGTGGGGC CCAGCG	-	10	20%	A222_S233	-	SLC45A3	Yes	Yes - IBD
x	x	x	chr1:210864874-210864874	C	T	18	22%	T	M	FAM71A	Yes	SNP
x		x	chr1:203211049-203211049	A	G	21	10%	E	G	NFASC	No	-
x		x	chr1:203294909-203294909	A	G	23	9%	T	A	CNTN2	No	-
x		x	chr1:203319711-203319711	A	G	34	6%	W	R	TMEM81	No	-
x		x	chr1:203405523-203405523	-	T	23	9%	D	N	DSTYK	No	-
x		x	chr1:203423176-203423176	A	G	33	6%	L	P	DSTYK	No	-

Table 19-continued.

x		x	chr1:203504860-203504860	-	G	18	11%	I	V	TMCC2	No	-
x		x	chr1:204006202-204006202	A	G	34	6%	V	A	RAB7L1	No	-
x		x	chr1:204842400-204842400	A	G	25	8%	F	S	LGTN	No	-
x		x	chr1:205108519-205108519	A	G	25	8%	E	G	IL20	No	-
x		x	chr1:205262123-205262124	TC	-	15	20%	GK	GX	C1orf116	No	-
x		x	chr1:205263109-205263109	T	C	11	18%	Q	R	C1orf116	No	-
x		x	chr1:205353009-205353010	AC	-	27	7%	T	X	C4BPA	No	-
x		x	chr1:205366860-205366860	C	T	27	7%	P	S	C4BPA	No	-
x		x	chr1:205720002-205720002	A	G	41	5%	I	V	CR2	No	-
x		x	chr1:205746001-205746001	T	C	24	8%	I	T	CR1		-
x		x	chr1:205807977-205807978	TG	-	44	5%	W	X	CR1	No	-

Table 19-continued.

x		x	chr1:205824728-205824728	-	G	36	6%	D	G	CR1	No	-
x		x	chr1:205937543-205937543	A	G	37	5%	Q	R	CR1L	No	-
x		x	chr1:207672272-207672272	-	GT	24	12%	AA	AC	LOC642587		-
x		x	chr1:208482286-208482286	-	A	33	6%	S	N	SERTAD4	No	-
x		x	chr1:209259202-209259202	T	C	27	11%	D	G	KCNH1	No	-
x		x	chr1:209593256-209593256	-	G	26	8%	N	D	TRAF5	No	-
x		x	chr1:210216573-210216573	G	A	59	3%	R	C	INTS7	No	-
x		x	chr1:210866121-210866125	AGGGA	GGG	13	31%	RE	GK	FAM71A	No	-
x		x	chr1:211482044-211482044	-	G	43	5%	T	A	RPS6KC1	No	-
	x		chr1:203204081-203204081	A	G	11	18%	D	G	NFASC	No	-
	x		chr1:203238391-203238391	C	AT	8	25%	P	I	NFASC	No	-

Table 19-continued.

	x		chr1:203541956-203541956	G	A	6	33%	P	S	NUAK2	No	-
	x		chr1:203766060-203766060	C	G	8	25%	Y	*	PCTK3	No	-
	x		chr1:203854793-203854793	A	G	19	11%	L	P	ELK4	No	-
	x		chr1:203955397-203955397	A	CAT	17	12%	LM	LN	NUCKS1	No	-
	x		chr1:204006134-204006134	T	C	17	12%	I	V	RAB7L1	No	-
	x		chr1:204693306-204693306	A	G	19	11%	I	V	SRGAP2	No	-
	x		chr1:204701371-204701371	A	G	14	14%	K	E	SRGAP2	No	-
	x		chr1:204719842-204719842	T	C	3	67%	L	P	IKBKE	No	-
	x		chr1:206336721-206336721	-	G	17	12%	V	A	PLXNA2	No	-
	x		chr1:208482275-208482275	A	-	20	15%	K	X	SERTAD4	No	-
	x		chr1:208602825-208602825	A	G	18	11%	E	G	HHAT	No	-

Table 19-continued.

	x		chr1:208827933-208827933	C	T	5	40%	T	I	HHAT	Yes	SNP
	x		chr1:209322750-209322750	C	ACT	23	9%	LA	LS	KCNH1	No	-
	x		chr1:209909158-209909158	A	G	21	10%	V	A	NEK2	No	-
	x		chr1:210340519-210340519	-	G	27	7%	T	A	DTL	No	-
	x		chr1:211125318-211125318	T	C	12	17%	S	P	FLVCR1	No	-

Table 20. 18 unique, novel, chromosome 1 synonymous variants. All variants are within linkage breakpoints.

Sample												
1020-15a	1020-15b	1020-18	Location	Ref Nuc	Var Nuc	Total Depth	Var Freq	Ref AA	Var AA	Gene	Sanger Validated	Familial segregation
x	x	x	chr1:203765311-203765311	C	G	17	59%	L	L	PCTK3	Yes	Yes
x		x	chr1:204079465-204079465	A	TG	20	10%	A	A	PM20D1	No	-
x		x	chr1:205715036-205715036	C	T	45	4%	Y	Y	CR2	No	-
x		x	chr1:205934427-205934427	A	G	36	6%	G	G	CR1L	No	-
x		x	chr1:206139040-206139040	T	C	25	8%	S	S	CD34	No	-
x		x	chr1:208479561-208479561	-	T	44	5%	S	S	SERTAD4	No	-
x		x	chr1:209913681-209913681	A	G	18	11%	L	L	NEK2	No	-
x		x	chr1:210216556-210216556	A	G	57	3%	A	A	INTS7	No	-
x		x	chr1:210588363-210588363	T	C	28	7%	N	N	PPP2R5A	No	-
x		x	chr1:211075923-211075923	T	C	19	11%	K	K	C1orf227	No	-
x		x	chr1:211240347-211240347	-	A	41	5%	D	D	ANGEL2	No	-
x		x	chr1:211407936-211407936	-	C	25	8%	S	S	RPS6KC1	No	-
	x		chr1:203539471-203539471	-	A	12	17%	P	P	NUAK2	No	-
	x		chr1:205172515-205172515	-	T	9	22%	A	A	PIGR	No	-
	x		chr1:205713670-205713670	T	C	17	12%	P	P	CR2	No	-
	x		chr1:208627440-208627440	G	A	18	11%	A	A	HHAT	No	-
	x		chr1:210340719-210340719	-	C	19	21%	L	L	DTL	No	-
	x		chr1:210685902-210685902	-	A	13	15%	E	E	NENF	No	-

Figure 13. Shared variant analysis between affected individuals.

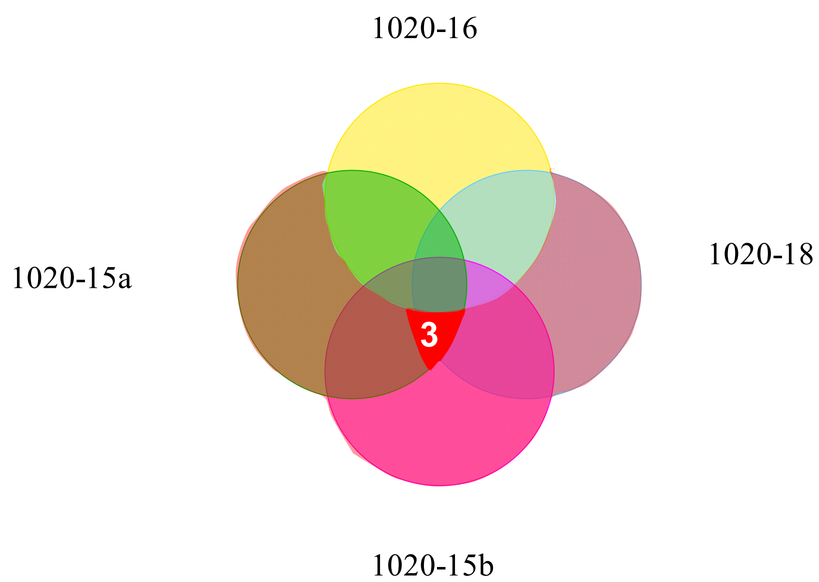


Figure 14. Familial segregation of variants validated by Sanger sequence.

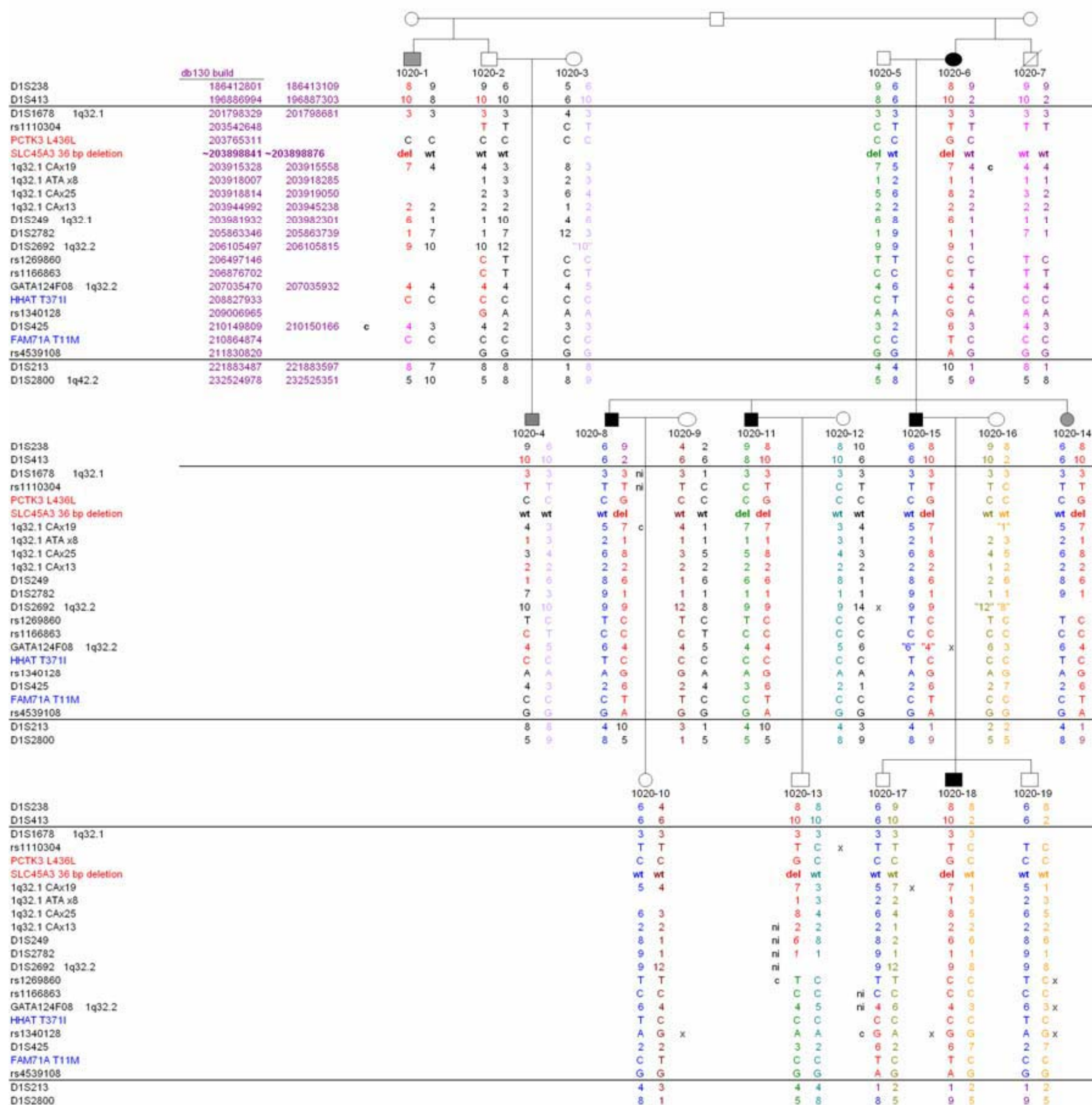


Figure 15. STRP markers spanning the *SLC45A3* candidate deletion in family 1020.

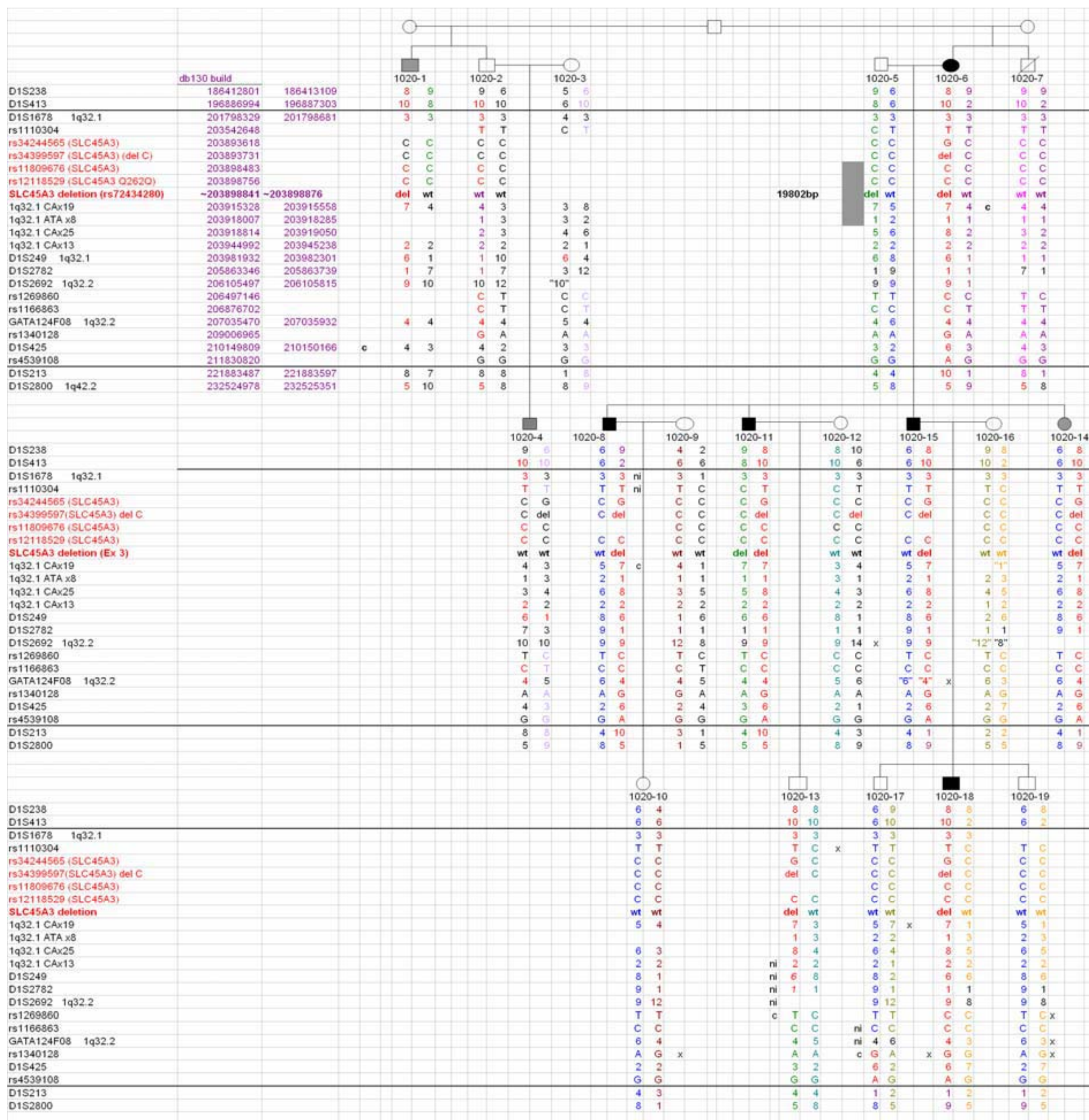
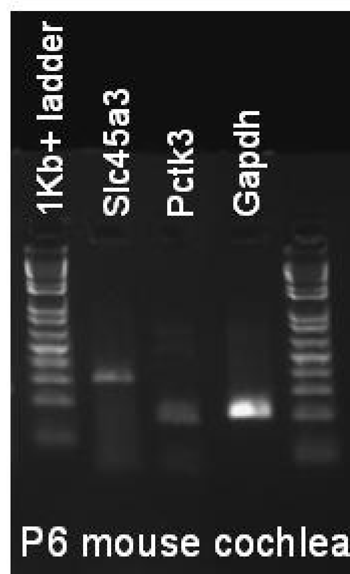


Figure 16. cDNA expression of candidate genes in P6 mouse cochlea.



Rare Variants

In *PCTK3*, 79 variants were genotyped and nine were identified in 1-2 patients and none of the controls (Table 21). Two of the nine variants were previously reported one of which is the synonymous change, R126R. Seven of the nine variants were novel, one of which was the non-synonymous change, R495Q, with a conservation score of 5. Two SNPs, one reported and one novel were each found in one control and none of the patients. The reported variant is rs17850752 which results in the non-synonymous change T196M with a conservation score of 8, and the novel variant was intronic. Conservation scores range from 1-9 with 1 indicating "not conserved" and 9 indicating "conserved". 95.3% and 96.0% of the *PCTK3* genotypes are complete for patients and controls, respectively.

In *SLC45A3*, 31 variants were genotyped and ten were identified in at least one patient and none of the controls (Table 22). Five of the 10 variants were previously reported, and three of those were coding, including C461C and L532L. The 36 bp deletion identified in family 1020 (A222_S233del) was identified in one patient and none of the controls (rs72434280; rs71152447). This deletion has not been identified in 409 controls screened in our lab. Five of the 10 variants were novel, one of which was the synonymous change V533V. Five variants were seen in one or more controls and none of the patients, all five variants were novel. Three of the five are non-synonymous including; R152W with a conservation score of 3, E227K with a conservation score of 1, and A528T with a conservation score of 6, and the other two variants are located in the 3'UTR. 96.0% and 94.6% of the *SLC45A3* genotypes are complete for patients and controls, respectively.

Common Variants

Several common variants have been found to be associated with MD. In *PCTK3* three variants had a significant χ^2 p-value <0.05 (Table 21). These variants include; rs71147749, a tri-nucleotide repeat located in the 5'UTR, rs28742123 also located in the 5'UTR (allelic χ^2 p-value 0.0014 and genotypic χ^2 p-value 0.00033), and a novel variant located in intron 9 (allelic χ^2 p-value 0.1126 and genotypic χ^2 p-value 0.0357). Following Bonferroni correction, only rs28742123 significantly differed between cases and controls (allelic χ^2 p-value 0.0042 and genotypic χ^2 p-value 0.0009). The Armitage trend test revealed a potential gender interaction for rs41264889 in intron 4, and with the rare T allele more common in males than females, linear χ^2 p-value= 0.0263 (OR [95%CI]= 3.9505 [1.0427-14.9673]. In *SLC45A3*, one reported variant differed significantly between cases and controls, rs41313722, located in intron 2 (allelic χ^2 p-value 0.0707 and genotypic χ^2 p-value 0.0233) (Table 22).

Table 21. *PCTK3* results for all variants genotyped in patients and controls. The conservation scores are also described as an exposed residue (e), or a buried residue (b) (Berezin, Glaser et al. 2004). NA= not applicable. P-values in bold are significant ($p<0.05$). Fluorescently labeled STRP markers are being optimized to correctly genotype rs71147749.

SNP	Amino Acid Change (Conservation)	MD Genotypes			Control Genotypes			Controls HWE χ^2 p-value	Allelic χ^2 p-value (*Yate's p-value)	Genotypic χ^2 p-value	OR (95% CI)	Gender interaction: linear χ^2 p-value (based on Armitage trend test)
rs71147749	-	8 (8/8)	8/het=3	9/9=37	8/8=0	9/9=12	10/10=23			**0.0000**		
rs28742123	-	71 (CC)	9 (CT)	0 (TT)	109 (CC)	0 (CT)	0 (TT)	NA	***0.0014**	**0.0003**	0.0000	0.6646
Novel	-	57 (TT)	46 (CT)	14 (CC)	43 (TT)	65 (CT)	15 (CC)	0.6627	0.1088	0.0781	1.3603 (0.9335-1.9823)	0.3072
rs4951006	-	91 (GG)	0 (CG)	0 (CC)	31 (GG)	0 (GC)	0 (CC)	NA	NA	NA	NA	NA
Novel	-	116 (GG)	8 (GA)	0 (AA)	119 (GG)	5 (GA)	0 (AA)	0.9746	0.3991	0.3926	0.6173 (0.1991-1.9138)	0.7762
Novel	-	121 (GG)	3 (GA)	0 (AA)	123 (GG)	1 (GA)	0 (AA)	0.9990	*0.6157	0.3135	0.3306 (0.0342-3.2007)	0.6769
rs71701308	N44_R526del	123 (wt)	0 (wt/del)	0 (del/del)	123 (wt)	0 (wt/del)	0 (del/del)	NA	NA	NA	NA	NA

Table 21-continued.

rs74141238	-	116 (CC)	6 (CT)	0 (TT)	118 (CC)	5 (CT)	0 (TT)	0.9744	0.7494	0.7471	0.8230 (0.2478-2.7331)	0.2758
rs35134237	G48S (2,b)	123 (GG)	0 (GA)	0 (AA)	124 (GG)	0 (AG)	0 (AA)	NA	NA	NA	NA	NA
rs35209603	S66R (3, e)	121 (GG)	2 (GA)	0 (AA)	122 (GG)	2 (GA)	0 (AA)	0.9959	*0.6213	1.0000	0.9919 (0.1386-7.0980)	0.1997
rs4623769	G67R (2, e)	116 (GG)	7 (AG)	0 (AA)	118 (GG)	6 (AG)	0 (AA)	0.9635	0.7667	0.7642	0.8465 (0.2804-2.5558)	0.1433
rs7529442	V92V	116 (GG)	7 (GA)	0 (AA)	116 (GG)	8 (GA)	0 (AA)	0.9355	0.8049	0.8018	1.1381 (0.4063-3.1881)	0.1433
rs28664770	-	123 (TT)	0 (TG)	0 (GG)	124 (TT)	0 (GT)	0 (GG)	NA	NA	NA	NA	NA
rs74141240	-	119 (TT)	4 (TC)	0 (CC)	122 (TT)	2 (TC)	0 (CC)	0.9959	*0.6740	0.4028	0.4919 (0.0893-2.7105)	0.2155
Novel	-	122 (CC)	1 (CG)	0 (GG)	124 (CC)	0 (CG)	0 (GG)	NA	*1.0	0.3144	0.0000	0.2642
rs61733643	R96R; R126R	122 (GG)	1 (GA)	0 (AA)	124 (GG)	0 (GA)	0 (AA)	NA	*1.0	0.3144	0.0000	0.2642

Table 21-continued.

rs41264889	-	111 (CC)	12 (CT)	0 (TT)	109 (CC)	15 (CT)	0 (TT)	0.7855	0.5674	0.5558	1.2554 (0.5752-2.7398)	**0.0263**
rs68168742	-	123 (wt/wt)	0 (wt/del)	0 (del/del)	124 (wt/wt)	0 (wt/del)	0 (del/del)	NA	NA	NA	NA	NA
rs66463634	-	120 (wt/wt)	0 (wt/del)	0 (del/del)	116 (wt/wt)	0 (wt/del)	0 (del/del)	NA	NA	NA	NA	NA
rs73074319	-	120 (GG)	0 (GA)	0 (AA)	117 (GG)	0 (GA)	0 (AA)	NA	NA	NA	NA	NA
rs12126241	-	109 (GG)	1 (GA)	1 (AA)	104 (GG)	13 (GA)	0 (AA)	0.8260	0.9287	0.5442	1.0362 (0.4700-2.2847)	0.6619
rs41310919	-	112 (GG)	9 (GA)	0 (AA)	115 (GG)	3 (GA)	0 (AA)	0.9904	*0.1563	0.0832	0.3333 (0.0891-1.2468)	0.4931
rs74141243	-	118 (CC)	0 (CT)	0 (TT)	113 (CC)	0 (CT)	0 (TT)	NA	NA	NA	NA	NA
rs17850752	T166M; T196M (8, e)	119 (CC)	0 (CT)	0 (TT)	112 (CC)	1 (CT)	0 (TT)	0.9989	*0.9748	0.0832	NA	NA
rs2297837	-	111 (GG)	8 (GA)	0 (AA)	104 (GG)	8 (GA)	0 (AA)	0.9286	0.9025	0.8993	1.0648 (0.3927-2.8871)	0.0814
Novel	-	118 (AA)	1 (AG)	0 (GG)	113 (AA)	0 (AG)	0 (GG)	NA	*0.9748	0.3287	0.0000	0.2706

Table 21-continued.

rs72477946	-	119 (wt/wt)	0 (wt/del)	0 (del/del)	112 (wt/wt)	0 (wt/del)	0 (del/del)	NA	NA	NA	NA	NA
rs67648691	-	119 (wt/wt)	0 (wt/del)	0 (del/del)	112 (wt/wt)	0 (wt/del)	0 (del/del)	NA	NA	NA	NA	NA
rs35202186	-	123 (GG)	0 (GT)	0 (TT)	124 (GG)	0 (GT)	0 (TT)	NA	NA	NA	NA	NA
Novel	-	123 (CC)	0 (CT)	0 (TT)	123 (CC)	1 (CT)	0 (TT)	0.9990	*1.0	0.3183	0.0000	NA
Novel	-	122 (GG)	1 (GT)	0 (TT)	124 (GG)	0 (GT)	0 (TT)	NA	*1.0	0.3144	0.0000	0.2642
rs67250950	-	123 (wt/wt)	0 (wt/del)	0 (del/del)	124 (wt/wt)	0 (wt/del)	0 (del/del)	NA	NA	NA	NA	NA
rs34804445	-	123 (wt/wt)	0 (wt/del)	0 (del/del)	124 (wt/wt)	0 (wt/del)	0 (del/del)	NA	NA	NA	NA	NA
rs71568054	-	116 (wt/wt)	7 (wt/del)	0 (del/del)	117 (wt/wt)	2 (wt/del)	0 (del/del)	0.9958	*0.1949	0.0993	0.2893 (0.0595-1.4072)	0.9189
rs1544154	-	123 (GG)	0 (GA)	0 (AA)	124 (GG)	0 (GA)	0 (AA)	NA	NA	NA	NA	NA
rs74141244	-	119 (CC)	0 (CT)	0 (TT)	122 (CC)	0 (CT)	0 (TT)	NA	NA	NA	NA	NA
rs2297836	-	98 (GG)	13 (GA)	0 (AA)	103 (GG)	10 (GA)	0 (AA)	0.8907	0.4925	0.4804	0.7443 (0.3194-1.7346)	0.4534

Table 21-continued.

Novel	-	95 (AA)	4 (AT)	0 (TT)	107 (AA)	0 (AT)	0 (TT)	NA	*0.1126	**0.0357**	0.0000	0.3796
rs2275348	-	65 (GG)	37 (GC)	5 (CC)	66 (GG)	36 (GC)	6 (CC)	0.9671	0.9483	0.9476	1.0152 (0.6437-1.6012)	0.8617
rs67754541	-	61 (wt/wt)	0 (wt/del)	0 (del/del)	107 (wt/wt)	0 (wt/del)	0 (del/del)	NA	NA	NA	NA	NA
rs2275347	-	104 (AA)	8 (AT)	2 (TT)	108 (AA)	13 (AT)	0 (TT)	0.8315	0.9563	0.2165	1.0218 (0.4562-2.2886)	0.8043
rs67860805	-	98 (wt/wt)	8 (GAAG/TTC)	2 (TTC/TTC)	107 (wt/wt)	13 (GAAG/TTC)	0 (TTC/TTC)	0.8302	0.9496	0.2274	0.9736 (0.4343-2.1821)	0.7711
rs2275346	-	102 (wt/wt)	8 (wt/del)	2 (del/del)	108 (wt/wt)	13 (wt/del)	0 (del/del)	0.8315	0.9944	0.2210	1.0029 (0.4477-2.2467)	0.7652
rs66519761	-	112 (wt/wt)	0 (wt/del)	0 (del/del)	121 (wt/wt)	0 (wt/del)	0 (del/del)	NA	NA	NA	NA	NA
rs12734053	-	118 (AA)	0 (AC)	0 (CC)	123 (AA)	0 (AC)	0 (CC)	NA	NA	NA	NA	NA
rs12408209	-	75 (CC)	35 (CG)	3 (GG)	79 (CC)	39 (CG)	5 (GG)	0.9989	0.6235	0.8199	1.1223 (0.7079-1.7793)	0.7604

Table 21-continued.

rs7531230	-	37 (CC)	59 (CT)	27 (TT)	41 (CC)	61 (CT)	21 (TT)	0.9892	0.3636	0.6100	0.8478 (0.5936-1.2108)	0.5223
Novel NM_212503.2:c.[1065-82C>T]	-	117 (CC)	6 (CT)	0 (TT)	118 (CC)	6 (CT)	0 (TT)	0.9635	0.9887	1.0000	0.9917 (0.3154-3.1183)	0.5653
Novel NM_212503.2:c.[1065-22C>T]	-	121 (CC)	1 (CT)	0 (TT)	122 (CC)	0 (CT)	0 (TT)	NA	*1.0	0.3163	0.0000	0.3629
rs61733640	A335T; A365T (9, b)	122 (GG)	0 (AG)	0 (AA)	121 (GG)	0 (GA)	0 (AA)	NA	NA	NA	NA	NA
Novel NM_212503.2:c.[1162+28C>G]	-	122 (CC)	1 (CT)	0 (TT)	120 (CC)	0 (CG)	0 (TT)	NA	*1.0	0.3222	0.0000	0.3665
Novel NM_212503.2:c.[1162+63G>A]	-	120 (GG)	3 (GA)	0 (AA)	117 (GG)	3 (GA)	0 (AA)	0.9906	*0.7034	0.9748	1.0253 (0.2049-5.1310)	0.4388
Novel	-	121 (CC)	2 (CT)	0 (TT)	117 (CC)	3 (CT)	0 (TT)	0.9906	*0.9748	0.6315	1.5443 (0.2557-9.3251)	0.1997

Table 21-continued.

rs4951235	-	112 (CC)	9 (CG)	2 (GG)	104 (CC)	16 (CG)	0 (GG)	0.7515	0.5202	0.1212	1.2802 (0.6020-2.7224)	0.6791
Novel	-	119 (CC)	0 (CA)	0 (AA)	124 (CC)	0 (CA)	0 (AA)	NA	NA	NA	NA	NA
Novel	L406L; L436L	119 (CC)	0 (CG)	0 (GG)	124 (CC)	0 (CG)	0 (GG)	NA	NA	NA	NA	NA
rs67752365	-	119 (wt/wt)	0 (wt/ins)	0 (ins/ins)	124 (wt/wt)	0 (wt/ins)	0 (ins/ins)	NA	NA	NA	NA	NA
rs67250826	-	119 (wt/wt)	0 (wt/ins)	0 (ins/ins)	124 (wt/wt)	0 (wt/ins)	0 (ins/ins)	NA	NA	NA	NA	NA
rs61822300	-	122 (CC)	0 (CT)	0 (TT)	122 (CC)	0 (CT)	0 (TT)	NA	NA	NA	NA	NA
rs12042887	-	99 (CC)	22 (CA)	2 (AA)	103 (CC)	20 (CA)	1 (AA)	0.9998	0.5240	0.7773	0.8237 (0.4532-1.4969)	0.5767
Novel	E417R; E447R (3, e)	123 (GG)	0 (GA)	0 (AA)	123 (GG)	0 (GA)	0 (AA)	NA	NA	NA	NA	NA
Novel	Y424X ; Y454X (6, b)	124 (CC)	0 (CG)	0 (GG)	124 (CC)	0 (CG)	0 (GG)	NA	NA	NA	NA	NA
rs56414525	R431R; R461R	122 (TT)	2 (TA)	0 (AA)	121 (TT)	3 (TA)	0 (AA)	0.9909	*1.0	0.6515	1.5061 (0.2495-9.0929)	0.8897

Table 21-continued.

rs55957903	-	124 (GG)	0 (GA)	0 (AA)	124 (GG)	0 (GA)	0 (AA)	NA	NA	NA	NA	NA
rs12035825	-	102 (GG)	20 (GA)	2 (AA)	105 (GG)	19 (GA)	0 (AA)	0.6742	0.4250	0.3554	0.7744 (0.4127-1.4532)	0.7273
rs6683498	-	124 (TT)	0 (TC)	0 (CC)	124 (TT)	0 (TC)	0 (CC)	NA	NA	NA	NA	NA
Novel	-	122 (CC)	0 (CT)	0 (TT)	122 (CC)	0 (CT)	0 (TT)	NA	NA	NA	NA	NA
rs41264895	-	74 (CC)	39 (CT)	9 (TT)	60 (CC)	55 (CT)	9 (TT)	0.8636	0.1265	0.1243	1.3685 (0.9145-2.0480)	0.7065
rs12748821	-	98 (TT)	23 (CT)	1 (CC)	103 (TT)	21 (CT)	0 (CC)	0.6152	0.4981	0.5491	0.8104 (0.4407-1.4902)	0.4574
rs11240505	-	103 (CC)	18 (CG)	2 (GG)	105 (CC)	19 (CG)	0 (GG)	0.6742	0.6054	0.3602	0.8448 (0.4451-1.6035)	0.7199
Novel	-	118 (CC)	3 (CT)	0 (TT)	119 (CC)	5 (CT)	0 (TT)	0.9746	*0.7483	0.4939	1.6392 (0.3874-6.9358)	0.6903
rs45495498	-	120 (CC)	1 (CG)	0 (GG)	123 (CC)	1 (CG)	0 (GG)	0.9990	*0.4893	1.0000	0.9757 (0.0607-15.6887)	0.3673

Table 21-continued.

Novel	R465Q; R495Q (5, e)	120 (GG)	1 (GA)	0 (AA)	124 (GG)	0 (GA)	0 (AA)	NA	*1.0	0.3104	0.0000	0.3673
rs11240507	-	72 (TT)	39 (TC)	10 (CC)	60 (TT)	55 (TC)	9 (CC)	0.8636	0.2073	0.1473	1.2938 (0.8666-1.9318)	0.6356
Novel	-	119 (CC)	2 (CG)	0 (GG)	122 (CC)	2 (CG)	0 (GG)	0.9959	*0.6330	0.9748	0.9756 (0.1363-6.9821)	0.8775
rs74142375	-	117 (TT)	4 (TA)	0 (AA)	119 (TT)	5 (TA)	0 (AA)	0.9746	*0.9748	0.7629	1.2243 (0.3248-4.6147)	0.8260
rs3838999	-	117 (wt/wt)	4 (wt/ins)	0 (ins/ins)	122 (wt/wt)	2 (wt/ins)	0 (ins/ins)	0.9959	*0.6596	0.3913	0.4837 (0.0878-2.6659)	0.2140
rs1042827	-	121 (GG)	0 (GA)	0 (AA)	124 (GG)	0 (GA)	0 (AA)	NA	NA	NA	NA	NA
rs3795547	-	115 (CC)	3 (CT)	0 (TT)	117 (CC)	5 (CT)	0 (TT)	0.9742	*0.7567	0.5019	1.6248 (0.3839-6.8765)	0.7262

Table 22. *SLC45A3* results for all variants genotyped in patients and controls. The conservation scores are also described as an exposed residue (e), or a buried residue (b) (Berezin, Glaser et al. 2004). NA= not applicable. P-values in bold are significant (p<0.05).

SNP	Amino Acid Change (Conservation, SIFT Prediction)	MD Genotypes			Control Genotypes			Controls HWE p-value	Allelic χ^2 p-value (*Yate's p-value)	Genotypic χ^2 p-value	OR (95% CI)	Gender interaction: linear χ^2 p-value
rs12060080	NA	CC=113	CG=3	TT=0	CC=116	CG=1	TT=0	0.9989	*0.6094	0.3090	0.3276 (0.0338-3.1728)	0.6850
Novel	NA	CC=116	CA=1	AA=0	CC=117	CA=0	AA=0	NA	*1.0	0.3163	0.0000	0.3690
Novel NM_033102.2:c.[-289G>A]	NA	GG=116	GA=2	AA=0	GG=114	GA=3	AA=0	0.9903	*1.0	0.6444	1.5195 (0.2516-9.1780)	0.1978
Novel NM_033102.2:c.[-246C>T]	NA	CC=115	CT=3	TT=0	CC=117	CT=0	TT=0	NA	*0.2497	**0.0826**	0.0000	0.4245
Novel NM_033102.2:c.[-199G>A]	NA	GG=116	GA=6	AA=0	GG=117	GA=7	AA=0	0.9504	0.8015	0.7988	1.1521 (0.3816-3.4788)	0.5531
Novel NM_033102.2:c.[-83G>A]	NA	GG=117	GA=5	AA=0	GG=118	GA=6	AA=0	0.9635	0.7813	0.7787	1.1851 (0.3569-3.9356)	0.2497

Table 22-continued.

Novel NM_033102.2:c.[-32C>T]	NA	CC=121	CT=1	TT=0	CC=124	CT=0	TT=0	NA	*1.0	0.3123	0.0000	0.3629
rs56927917	NA	AA=119	AG=3	GG=0	AA=119	AG=5	GG=0	0.9746	*0.7390	0.4866	1.6529 (0.3907-6.9936)	0.4649
rs41313722	NA	CC=118	CT=5	TT=0	CC=124	CT=0	TT=0	NA	***0.0707**	**0.0233**	0.0000	0.8286
Novel NM_033102.2:c.[454C>T]	R152W (Arg152Trp) (3, e, Tolerate)	CC=123	CT=0	TT=0	CC=123	CT=1	TT=0	0.9990	*1.0	0.3183		NA
rs72434280; rs71152447	A222_S233del (3e, 2e, 2e, 5e, 1e…)	wt=120	wt/del=1	del/del=0	wt/wt=124	wt/del=0	del/del=0	NA	*1.0	0.3104		0.2633
Novel	E227K (1, e, Tolerate)	GG=120	GA=0	AA=0	GG=123	GA=1	AA=0	0.9990	*1.0	0.3242		NA
rs12118529	Q262Q	GG=121	GA=0	AA=0	GG=124	GA=0	AA=0	NA	NA	NA	NA	NA
rs74143150	NA	CC=121	CT=0	TT=0	CC=124	CT=0	TT=0	NA	NA	NA	NA	NA
rs11809676	NA	CC=105	CT=0	TT=0	CC=117	CT=0	TT=0	NA	NA	NA	NA	NA
rs16856106	T385T	CC=118	CT=6	TT=0	CC=113	CT=7	TT=0	0.9488	0.7330	0.7290	1.2117 (0.4013-3.6592)	0.5506
rs35201492	C461C	CC=119	CT=1	TT=0	CC=120	CT=0	TT=0	NA	*1.0	0.3163	0.0000	0.2589

Table 22-continued.

Novel NM_033102.2:c.[1 408G>A]	V470I (5, b, Tolerate)	GG=117	GA=3	AA=0	GG=116	GA=4	AA=0	0.9832	*1.0	0.7014	1.3390 (0.2965- 6.0478)	0.1187
Novel	A528T (6, b, Tolerate)	GG=114	GA=0	AA=0	GG=116	GA=2	AA=0	0.9957	*0.4939	0.1627		NA
rs7540439	L532L	GG=119	GC=1	CC=0	GG=119	GC=0	CC=0	NA	*1.0	0.3183	0.0000	0.2508
Novel NM_033102.2:c.[1 599C>T]	V533V	CC=117	CT=1	TT=0	CC=118	CT=0	TT=0	NA	*1.0	0.3163	0.0000	0.2497
rs55795602	NA	TT=122	TC=1	CC=0	TT=124	TC=0	CC=0	NA	*1.0	0.3144	0.0000	0.2642
rs17347787	NA	CC=100	CT=22	TT=1	CC=102	CT=19	TT=0	0.6671	0.4580	0.5425	0.7881 (0.4198- 1.4796)	0.5806
Novel	NA	CC=124	CT=0	TT=0	CC=122	CT=1	TT=0	0.9990	*1.0	0.3144		NA
Novel	NA	GG=124	GA=0	AA=0	GG=122	GA=1	AA=0	0.9990	*1.0	0.3144		NA
rs2275753	NA	CC=118	CT=5	TT=0	CC=114	CT=5	TT=0	0.9735	0.9579	0.9563	1.0343 (0.2956- 3.6197)	0.1052
rs34399597	NA	wt/wt=11 1	wt/del=11	del/del =0	wt/wt=10 9	wt/del=15	del/del=0	0.7855	0.4452	0.4322	1.3636 (0.6155- 3.0316)	0.2159
rs71984296	NA	wt/wt=12 3	wt/del=0	del/del =0	wt/wt=12 4	wt/del=0	del/del=0	NA	NA	NA	NA	NA

Table 22-continued.

rs34244565	NA	GG=116	GC=0	CC=2	GG=112	GC=0	CC=0	NA	0.1458	0.1664	0.0000	NA
rs35504176	NA	CC=113	CT=0	TT=0	CC=121	CT=0	TT=0	NA	NA	NA	NA	NA
rs74143149	NA	TT=113	TG=0	GG=0	TT=104	TG=0	GG=0	NA	NA	NA	NA	NA

Discussion

Novel MD Locus

We have identified a novel locus for familial MD and within the linked region, two candidate causative variants. The first variant, a synonymous single nucleotide variant (SNV) - *PCTK3* (NM_212503)*,* exon 13, C→G, L436L, segregates with MD in the family. *PCTK3* is also known as Cell division protein kinase 18 (*CDK18*) and has multiple isoforms, the longest has16 exons that encode PCTAIRE 3, a 504-amino acid protein kinase. *PCKT3* expressed sequence tags (ESTs) are found in brain, bladder, eye, ovary, larynx, heart, thymus, nerve, adrenal gland, kidney, and skin and expressed in lower levels in additional tissues (http://www.ncbi.nlm.nih.gov/unigene) (Sayers, Barrett et al. 2010). Although its function is unknown, PCTAIRE 3 may alter tau phosphorylation in Alzheimer's disease (Herskovits and Davies 2006; Cole 2009). The synonymous change we identified is predicted to alter splicing *in silico* (Table 23).

Table 23. Predicted ESE alterations by *PCTK3* L436L, (http://rulai.cshl.edu/cgi-bin/tools/ESE3) (Cartegni, Wang et al. 2003; Smith, Zhang et al. 2006). The SNV is highlighted in red.

SC35 Thr=2.383			Srp40 Thr=2.67			SRp55 Thr=2.676		
Pos	Motif	Score	Pos	Motif	Score	Pos	Motif	Score
17	TGCT**C**CTG	4.1711	19	CT**C**CTGG	3.0143			
17	TGCT**G**CTG	3.2734	19	CT**G**CTGG	2.8011	17	TGCT**G**C	3.0180

The second identified variant is a 36-bp deletion in exon 3 of *SLC45A3* (NM_033102) that results in a 12-amino acid in-frame deletion (A222-S233). At the time of this study, this variant was novel, but it has subsequently been assigned two rs

numbers in dbSNP130, rs72434280 and rs71152447. Population frequency information is not yet available however we did not find this variant in 409 controls (818 chromosomes).

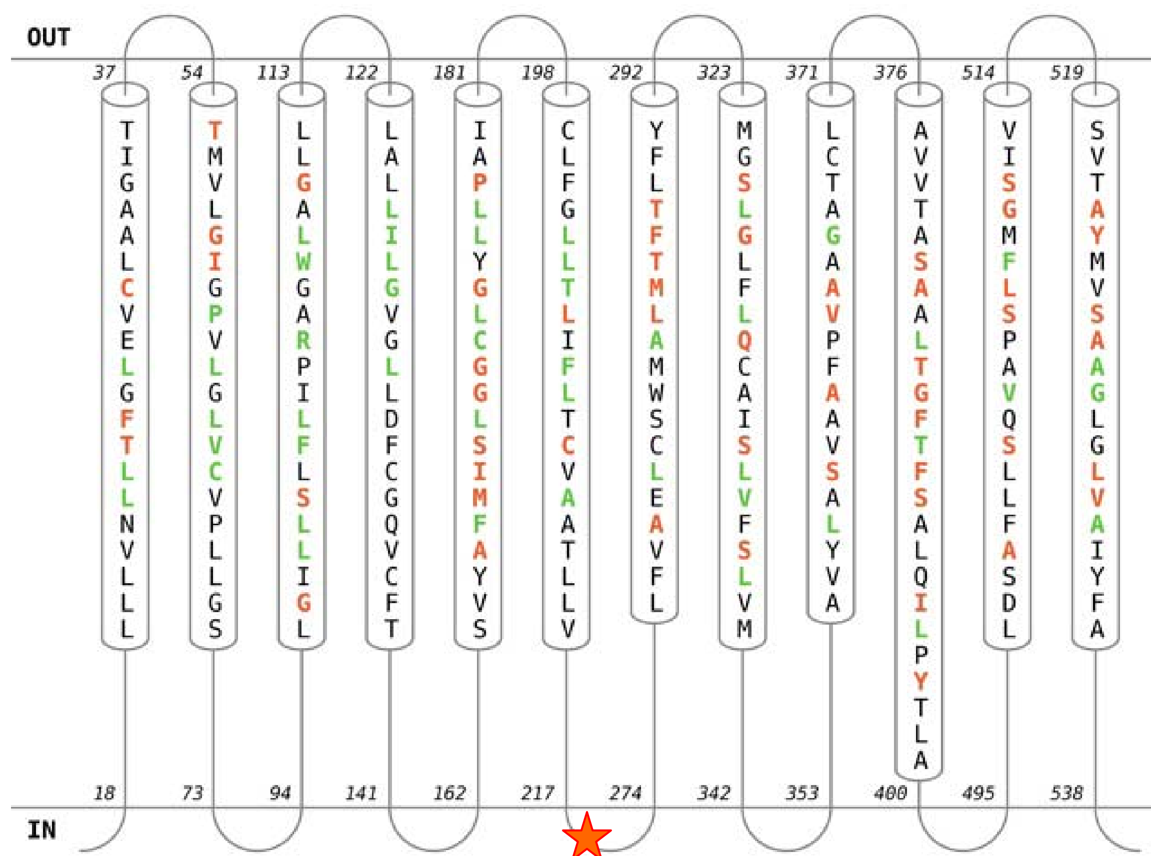
Unexpectedly, the proband's brother (1020-11) is homozygous for this deletion however his MD was early in onset and reports severe disease (Table 15). Given the apparent rarity of this deletion, we considered a possible distant founder effect and documented a region of parental autozygosity by descent around the deletion. The families of the probands' parents both originate from the same Basque region of Spain. It is noteworthy that the proband's father has no self-reported symptoms of disease, however he has not been examined. The penetrance of MD is also only 60-90% (Morrison 1995; Frykholm, Larsen et al. 2006).

SLC45A3 has five exons and encodes the 553-amino acid protein, Prostein. *SLC45A3* ESTs are found in prostate, nerve, intestine, lung, ascites, trachea, vascular, spleen, embryonic tissue, mouth, skin, pancreas, brain, muscle, testis, mammary gland and liver (http://www.ncbi.nlm.nih.gov/unigene) (Sayers, Barrett et al. 2010). It has 12 predicted transmembrane helices, with (A222_S233) being in the cytosol between transmembrane spans 6 and 7 (Figure 17). The first deleted amino acid (A222) is the last amino acid of a predicted Na-dicarboxylate_symporter domain (amino acids 202-222). Although very little is known about prostein, it is believed to be a carbohydrate transporter and is known to be androgen responsive (i.e. up-regulated by androgens) in the prostate (Rickman, Pflueger et al. 2009). *SLC45A3* has been found to be a 5' fusion partner in ETS gene fusions in prostate cancer (Rickman, Pflueger et al. 2009).

The proband's father was recently diagnosed with prostate cancer. It is unlikely that a prostate cancer gene was identified in this family instead of a gene for MD for several reasons; the older age of the proband's father (early 80s) at time of diagnosis; prostate cancer is highly prevalent in the general population; only one family member is

reported to be diagnosed with prostate cancer. However, the family should be counseled to pursue prostate cancer screening as recommended for the general population.

Figure 17. Predicted *SLC45A3* domains. Predicted by the membrane topology prediction program, RHYTHM (http://proteinformatics.charite.de/rhythm/)(Rose, Lorenzen et al. 2009). The familial 36 bp deletion (A222_S233) is indicated by a star.



Neither *SLC45A3* nor *PCTK3* would have been selected as candidate genes illustrating the utility of this type of experimental approach. We must now determine which variant is the causal variant in this family. The variant in *PCTK3*, L436L, is the preferred candidate based on segregation with disease in the family, but is only predicted to possibly alter exon splicing. However, the A222_S233 deletion in *SLC45A3*, deletes 12 amino acids and is therefore preferential as it is more likely to alter protein function. To determine which variant and gene is causing MD in the family we next tested cDNA expression of both genes in the inner ear. Both *Pctk3* and *Slc45a3* were expressed in the P6 mouse cochlea (Figure 16). We are currently optimizing the primer conditions on human fibroblast cDNA to test the cDNA expression of both genes in adult human endolymphatic sac.

In the near future additional characterization of both genes by immunohistochemistry (IHC) and *in situ* hybridization (ISH) will be performed to further characterize their expression in the inner ear. Since endolymph is produced by the cells of the stria vascularis, and resorbed by the endolymphatic sac, expression in either the cells of the stria vascularis and/or in the endolymphatic sac would be highly suggestive of a role for either gene in the pathogenesis of MD. It is possible that one gene will be expressed in these tissues, both genes will be expressed in these tissues, or neither gene will be expressed. Expression in the outer or inner hair cells, spiral ligament or spiral ganglion will be considered a negative result, although expression in these or other cell types of the cochlea does not rule these genes out as candidate for MD since we do not know the initiating factor for disease.

Rare Variants and Common Variants in Ménière's Disease

The identification of putative disease causing variant in *PCTK3* and *SLC45A3* in family 1020 has lead us to investigate if rare and or common variants in either gene are associated with MD in a cohort of sporadic MD patients. *PCTK3* encodes PCTAIRE 3 a

non-receptor serine/threonine protein kinase possibly involved with protein phosphyorlyation, cell cycle control and mitosis according to PANTHER classification. *SLC45A3* encodes Prostein a putative carbohydrate transporter. Since little is known about the function of either gene functional studies to determine the role or either gene in the development of MD are difficult. We hypothesize that if either gene is involved with the development of familial MD it will also be involved with the development of sporadic MD. In individuals with sporadic MD there will be rare and common variants associated with the disease causing gene. A similar scenario was recently identified for a gene involved with the development of stuttering (Kang, Riazuddin et al. 2010). This was a feasible option as we have a large cohort of individuals diagnosed with MD as well as a cohort of matched controls. By analyzing rare and common variants we were able to test the Common disease Rare Variant hypothesis (CDRV) in which multiple rare variants with moderate to high penetrance cause complex disease. We were also able to test the Common disease Common Variant hypothesis (CDCV), in which common variants with modest effects cause common disease. Our hypothesis was a causative gene in a rare familial form of the disease will also be involved with the development of the more common sporadic form of the disease.

Rare Variants

In *PCTK3*, nine rare variants were identified in patients, including the previously reported SNV, R126R which is not predicted to affect splicing, and seven were novel including a non-synonymous SNV, R495Q (Table 21). In *SLC45A3*, ten rare variants were identified in patients, of the five previously reported variants, C461C and L532L both may alter exon splicing (Table 22). The 36 bp deletion identified in the Chilean family in this study was also identified in one singleton patient but not in 818 control chromosomes. Of the novel rare variants identified in MD patients, one was coding, V533V, which is predicted to possibly alter splicing.

Common Variants

Although sequencing has not been completed in all of the patients and controls several common variants have been found to be associated with MD. In *PCTK3* three variants had a significant χ^2 p-value <0.05; rs71147749 (5'UTR), rs28742123 (5'UTR), and a novel intron 9 SNV (Table 21). The first two variants are located beside each other and are most likely in linkage disequilibrium, and fluorescently labeled STRP primers are being optimized to accurately genotype the tri-nucleotide repeat. Neither variant is predicted to alter a transcription factor binding site. However, following corrections for multiple testing, only rs28742123 remained significantly different between MD patients and controls. Interestingly, for rs41264889 in intron 4, the T allele (rare allele) was found more frequently in male patients than female patients. Due to the multiple isoforms of PCTK3, this variant is located in the promoter, an intron (intronic splice enhancer), or downstream. The rare T allele is predicted to remove a GATA-X transcription factor binding site (http://www.cbrc.jp/research/db/TFSEARCH.html) (Heinemeyer, Wingender et al. 1998). The HapMap haploblocks for *PCTK3* and *SLC45A3* are not well defined (Figures 18 and 19**,** respectively). Haploblocks will be created with the patients and controls from the current study.

In *SLC45A3*, rs41313722 in intron 2 was significantly different between cases and controls (Table 22). The rare A allele is of this variant is predicted to create a CdxA transcription factor binding site (http://www.cbrc.jp/research/db/TFSEARCH.html) (Heinemeyer, Wingender et al. 1998). Due to the two isoforms of *SLC45A3* this transcription factor binding site can also be a promoter regulatory region or an intronic splice enhancer.

In summary, both *PCTK3* and *SLC45A3* had rare and common variants associated with MD in a singleton population. Since the sequencing of all cases and all controls is not yet complete additional rare or common variants may be identified. Sequencing 124 MD patients and 124 matched controls has the power to identify one rare variant with a

frequency of 0.001, if 10 variants exist in a gene and the results from this study are consistent with that prediction (Li and Leal 2009). However, we are still unclear as to which is the pathogenic gene in the family and in sporadic MD. It could be the only one gene is involved with the development of MD, or both genes are involved, or neither gene is involved with the development of MD.

In order to elucidate the pathogenic gene(s) further studies are necessary. First, immunohistochemistry (IHC) and in situ hybridization (ISH) can pinpoint where these genes are expressed in the inner ear. In addition, it still needs to be determined if either gene is expressed in the human endolymphatic sac. In the near future we will screen a second cohort of 124 MD patients matched by age and gender to the first cohort of MD patients already screened in this study, as well as 124 additional matched controls. Doubling the population size will increase our power and thereby ability to detect both rare and common variants for MD and in combination with the inner ear characterization by IHC, help clarify the disease causing gene in the Chilean family and sporadic MD.

Both variants have the potential to alter splicing and thereby protein structure and function, therefore studies of splicing affects either *in vitro* using fibroblast cells, or cDNA sequencing of the transcriptome may help answer these questions. Sequencing the transcriptome is a highly attractive option as it has previously identified novel and alternate exons, alternate start sites and un-translated regions, as well as tissue specific isoforms (Sugarbaker, Richards et al. 2008; Sultan, Schulz et al. 2008; Maher, Kumar-Sinha et al. 2009; Monaghan, Epp et al. 2009; Verlaan, Ge et al. 2009; Zhao, Caballero et al. 2009). If the same three individuals were used for transcriptome sequencing (1020-15, 1020-16, 1020-18), the results could be compared to the genomic exon sequences for the 1q32.1-1q32.3 region, and specifically these two candidate genes. In addition, sequencing the family trio would simplify data interpretation as the causative variant must segregate with disease in the trio.

It is possible neither gene is the causative gene for MD in this family. This is feasible as 129 kb (8.3%) of the coding sequence in the 1q32.1-1q32.3 interval was not included in the array design. Therefore, the causative variant may have been missed, in which case we need to sequence the 129 kb not previously sequenced. Another explanation may be 1020-4 is not a phenocopy but is affected with MD and the linkage analysis is incorrect. This is possible as we have not examined this individual ourselves and cannot be 100% confident in the symptom report. However, this alternative is unlikely as there were too few alleles shared between this individual and the other affected individuals to include him in the linkage analysis. Alternatively, traditional genomic alterations may not be directly responsible for the disease but rather an epigenetic modification may be cause MD in this family.

In conclusion, this study has identified a novel Ménière's disease locus on 1q32.1-1q32.3. This study is exciting because it is the first study we are aware of that combines linkage data from a large family along with targeted capture sequence data (Biesecker 2010). As with previous attempts for MD, it is difficult to identify a specific causative gene for this rare and complex disease. However, two excellent candidate genes and candidate variants have been identified in this family as well as rare and common variants in sporadic MD patients, therefore both genes warrant further investigation into their role in the pathogenesis of Ménière’s disease.

Figure 18. HapMap CEU LD Plot and Haploblock for *PCTK3*.

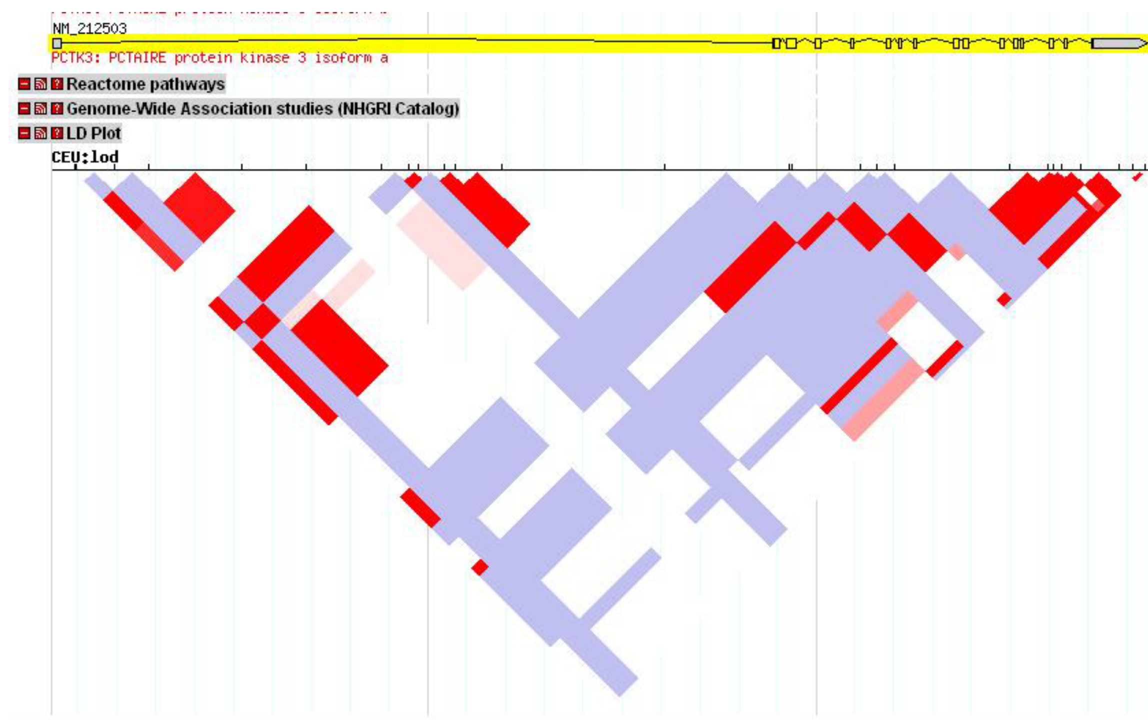
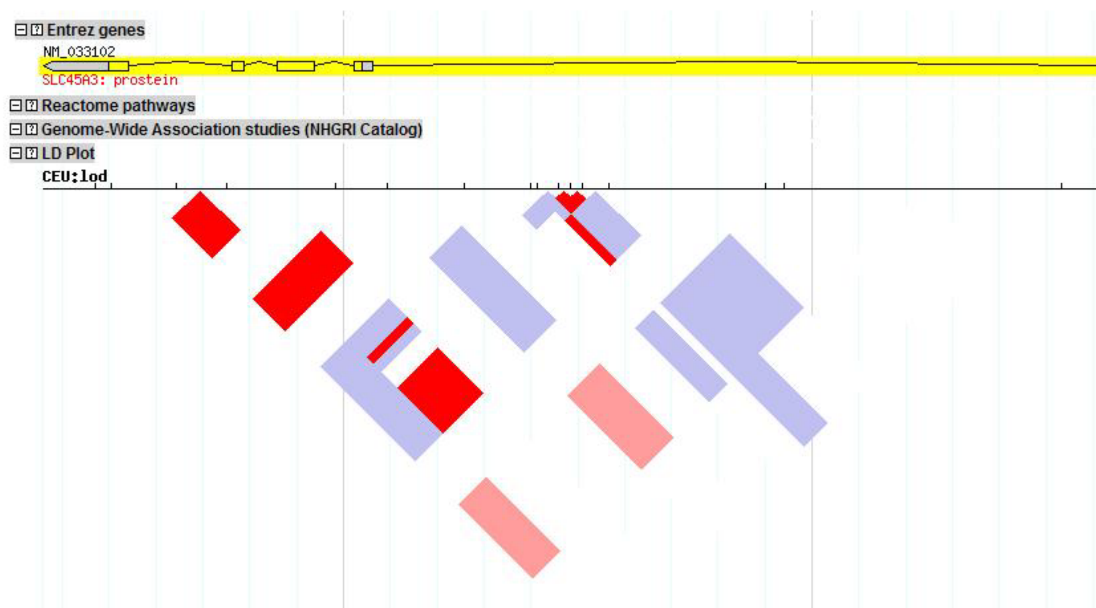


Figure 19. HapMap CEU LD Plot and Haploblock for *SLC45A3*.



CHAPTER V

CONCLUSIONS

Genetic Studies

MD is a complex disorder of the inner ear that is difficult to accurately diagnosis and treat. It is characterized by endolymphatic hydrops and appears to involve both environmental factors such as stress and high salt diet as well as genetic predisposition factors. Given that a naturally occurring animal does not exist for MD, several studies have attempted to identify genetic factors important in MD through the use of families segregating the disease in an autosomal dominant manner, however the paucity of large families segregating the disease suggests MD is a complex disease. Since most MD patients are sporadic, cohorts of singleton individuals have been used in several small association studies in attempt to identify genetic variants associated with the disease. To date a disease-causing gene in persons with MD as defined by the AAO-HNS has not been identified across populations. Many of these previous attempts have been unsuccessful in persons with definite MD largely due to the fact that families of sufficient size as well as cohorts of singleton individuals, to generate meaningful linkage results are extremely rare. With the advent of new technologies, sequencing all known and hypothetical exons and regulatory regions in a linked candidate region may help elucidate a pathogenic mutation. In addition, copy number variations, gene rearrangements, as well as epigenetic modifications may be worth investigating as potential sources of pathogenic variation. Although there are multiple small associations published, analysis of larger well defined cohorts of patients and matched controls are necessary to validate these preliminary results before any conclusions can be made regarding these genes and the pathogenesis of MD. Once a gene has been identified across populations, if an animal model is created it should be careful phenotyped at multiple ages and under

stressful conditions, as well as histological analysis performed to verify the presence of endolymphatic hydrops to determine if it is an accurate model for MD.

Replication of Case-Control Candidate Gene Association Studies

Evaluating and interpreting case-control candidate gene association studies can be challenging as the criteria for a good association study design are numerous and meaningful results can be difficult to interpret. Candidate gene association study design requires *a priori* knowledge about disease pathogenesis in order to select appropriate candidate genes. This is challenging for studies regarding MD as very little is known about the pathogenesis of MD. We attempted and were unable to replicate two candidate gene association studies. The first attempt was to replicate the findings of two SNPs in *KCNE1* and *KCNE3* associated with MD in a Japanese cohort reported by Doi and colleagues. The previously reported SNPs were not associated with MD in our Caucasian cohort and allele frequencies did not differ between the Japanese and Caucasian MD patients. This lead us to further investigate why the replication attempt was unsuccessful. After considering the factors critical to a successful association study design we concluded the controls in the original Japanese study were not appropriate for the patient population. To be sure the lack of replication was not due to ethnic allele frequencies differences we screened the entire coding region of both genes, but we did not find any variants associated with MD in a Caucasian population suggesting these genes are not involved with the disease in Caucasians.

The next attempt to replicate an association was at the request of a potential collaborator who asked us to replicate his preliminary association between *iNOS* and a MD Spanish population. We were unable to replicate this association. The original study had a high percentage of bilateral MD patients which is unusual and may be a reflection of inappropriate diagnosis and poor definition of a patient cohort. The controls were also

not matched in a traditional manner therefore the association may be spurious due to poorly matched controls.

Both of these replication attempts emphasize the need for carefully designed studies as many factors can lead to spurious results. In addition, replication of associations in additional large cohorts is necessary to elucidate true and spurious associations prior to genetic testing for MD. Although there have been several associations reported for MD many of these studies have small cohorts and inadequate study design to detect a true association and are probably spurious results. Therefore, we believe a true genetic predisposition to MD had not yet been identified prior to the work described in Chapters 3 and 4 of this thesis.

AQP4 and Syndromic MD

AQP4 is expressed in multiple places in the cochlea as well as the endolymphatic sac and although the deficient mice have hearing loss, to date a disease causing mutation has not been identified in humans. We selected *AQP4* to screen in a singleton cohort of MD patients and matched controls due to its role in fluid regulation and the importance of maintenance of endolymph in the cochlea for hearing. Although we did not find any common variants associated with MD in our population we identified a rare variant in an individual whom we thought was a sporadic case. Upon counseling this individual and obtaining a detailed family history we became suspicious of syndromic MD in the family. Subsequently a second patient was found to have the same rare variant, M224T. Again, upon obtaining a detailed family history, syndromic MD seemed possible in the second family. We first investigated if the variant could be due to a common founder, but this does not seem likely. A third individual with a 3'UTR rare variant was identified and also has bowel disease. These families need to evaluated clinically and accurately categorized as possible, probable, or definite MD, as well as evaluated for other features which may be associated with MD in these families such as hypertension, migraine, and

bowel disease. The finding of syndromic MD in two families is exciting as a gene for syndromic MD has not been reported in the literature. Careful evaluation and further studies of these families may lead to novel therapeutic options for MD with regards to fluid maintenance. I hypothesize *AQP4* is not solely causing MD in these individuals as *AQP4* is highly expressed throughout the body and one might expect a more severe phenotype. Reasons why this candidate gene association study may have been more successful than prior candidate gene association studies could be due to a large carefully defined patient cohort as well as a large well matched control cohort. In addition, expression of *AQP4* was previously well defined in the ear and the general function of the aquaporins family is well understood. The finding of hearing loss in the *Aqp4* deficient mouse further supports a role for this gene in the ear. All of these factors together make selection of an appropriate candidate gene easier combined with a well characterized patient cohort increase the likelihood of identifying a causative gene for MD.

A Novel Locus for MD

Our next attempt to identify a genetic component to MD also resulted in exciting findings. We were able to identify a large Chilean family segregating MD in an autosomal dominant manner over three generations and through the use of a genome wide linkage study identify a novel locus for MD on 1q32.1-1q32.3. The interval contained over 100 genes, and based on the knowledge we had gained with candidate gene studies for MD, we decided it would be extremely difficult to select an appropriate candidate gene. Instead we used a targeted capture approach along with pyrosequencing to screen all of the known and predicted genes in the interval. The results proved us correct in that we would have never selected the final two candidate genes, *PCTK3* and *SLC45A3*, as very little is known about the function of either gene and neither gene was known to be expressed in the ear. Although the SNV in *PCTK3* segregates perfectly with

disease it is a synonymous change and may or may not alter splicing. The 36 bp deletion in *SLC45A3* results in a deletion of 12 amino acids which is suggestive of an altered protein but the deletion is found in the non manifesting father of the proband. Although, we have not yet determined which gene in the novel locus is responsible for MD in the family, but we have ruled out a large portion of the locus by targeted exon capture and pyrosequencing of all known and hypothetical exons in the interval. We found cDNA of both *Pctk3* and *Slc45a3* to be expressed in the mouse cochlea and are currently screening both genes in human endolymphatic sac. Further characterization of both genes by IHC will be performed in the near future.

We subsequently screened a singleton MD population and matched controls in attempt to elucidate which of the two genes might be involved with the development of MD. Traditionally, additional families may have been screened but due to the paucity of families segregating MD we decided to instead screen a large cohort of sporadic MD patients. Our hypothesis was that a gene responsible for familial MD would also be involved with the development of sporadic MD and rare and common variants would be associated with the causative gene in a sporadic MD population. We did find both rare and common variants in both *PCTK3* and *SLC45A3* in our singleton cohort. We will be screening an additional 124 MD patients matched to our first singleton cohort as well as 124 matched controls for additional rare and common variants in both genes to further define rare and common variants in these genes and elucidate which may be involved with the development of MD.

In attempt to clarify which gene is responsible for MD in the family, we can also try to collect additional affected and unaffected family members to investigate segregation of the putative causal variants in the extended family. This option will receive lower priority as the adult onset nature of MD, along with reduced penetrance and potential phenocopies can complicate data interpretation. Alternatively, we can attempt

to better characterize MD symptoms in the family by physical exam, patient history, and audiometric testing.

Although an animal model of either gene could help us learn more about the function of these genes, a mouse or zebrafish model is not currently available for either gene, although knockout ES cells for *Slc45a3* will be available soon. An animal model will be critical in determining if alterations of these genes can cause MD symptoms under normal or stressful conditions and for further characterization of both genes.

Both the synonymous variant in *PCTK3* and the deletion in *SLC45A3* have the potential to alter splicing. We could perform in vitro splicing assays to confirm these possibilities. Alternatively, we can sequence the cDNA or transcriptome. To sequence the transcriptome, 200ng of RNA obtained from peripheral blood or skin biopsy will be obtained from the same family trio (1020-15, 1020-16, 1020-18), and sent to the 454 Life Sciences Service Center (454 Sequencing Center, 1 Commercial Street, Branford, CT 06405 USA) for cDNA sequencing. For each sample a list of expressed genes, novel transcripts, alternate isoforms, un-translated regions (UTRs), and variants will be compiled with particular attention to *PCTK3* and *SLC45A3*. In addition, lists of transcripts and variants in the two affected samples (1020-15 and 1020-18) but not present in the unaffected sample (1020-16) will be compiled. For variants, concordance between genomic sequence data and cDNA sequence will be interrogated. In addition, whether *PCTK3* L436L and *SLC45A3* A222-S233 alter splicing will be investigated. Any novel isoforms or transcripts will be verified by quantitative PCR (qPCR) in patient and control samples. Combining deep sequencing genomic data with transcriptome data may allow for the detection of intronic variants which alter splicing resulting in abnormal mRNA isoforms (Biesecker 2010).

An alternative option is to shotgun sequence the proteome to look for alterations in protein sequence in *PCTK3* and *SLC45A3*. Although this is a powerful and relatively inexpensive tool, especially when combined with genome and transcriptome sequencing,

it is a less attractive first option for us to pursue as we cannot obtain inner ear tissue from the family. We could alternatively obtain a skin biopsy from family members (1020-15, 1020-16, 1020-18). This would allow us to determine if the putative splice variant in *PCKT3* and deletion in *SLC45A3* alter protein sequence and possibly protein structure and function.

The results of this study are exciting as it is the first to identify novel locus with large family as well as putative causative genes with targeted capture, and then apply those results to a larger singleton cohort of patients. Since a single investigation is not likely to provide the answer regarding the pathogenic gene, we will integrate the information from the family study, the singleton association study, expression studies and possibly the transcriptome study to determine the which disease is responsible for MD.

The results of this thesis have taught us that identification of an appropriate candidate gene is difficult, and definition of patient and control cohorts is critical to association study design. In addition, it is important to ask patients about a family history of MD and be aware of potential cases of partial disease. Once families segregating the disease are identified careful phenotyping should be performed on all individuals in the family before a genome wide linkage or candidate gene association study performed. Once a disease gene is identified in a family, additional families as well as singleton patients should be screened for potential disease causing variants to prove the gene is pathogenic. Finally, combining traditional gene identification methods with new technologies is an efficient manner to identify causative genes in complex diseases such as MD.

In the future I would like to write a grant to sequence cDNA obtained from human endolymphatic sac tissue of MD patients and unaffected individuals to identify endolymphatic sac specific transcripts. This would be helpful not only to identifying genes that have a role the pathophysiology of MD but also to other hearing and balance disorders. Alternatively, endolymphatic sac cDNA obtained from unrelated MD (n=8)

and unaffected (n=8) individuals could be sequenced. This would allow us to look for protein sequences unique to the endolymphatic sac and identify potential altered protein sequences in MD patients which would be candidates for rare and common variant gene screening as well as pathways which may be involved with the development of MD.

A second aim could be to sequence cDNA obtained from blood and endolymphatic sac tissue from the same patient(s). As MD is a complex disease involving both genetic and environmental components, one could argue somatic mutations may develop in MD patients which increase disease susceptibility and ultimate development of disease. Identification of putative somatic variants in multiple patients could result in a list of candidate genes and pathways for further investigation to help define environmental triggers to MD and potential treatments. This method is commonly being used in the field of cancer genetics and given the complex and adult onset of both MD and cancer a similar approach may be helpful to learn more about MD (Sugarbaker, Richards et al. 2008; Prickett, Agrawal et al. 2009).

A third aim could be to sequence the exomes and transcriptomes of 100 MD patients to identify commonly altered genes for further study. The results of this thesis have proven identification of genetic components to complex diseases such as MD are difficult using traditional methods and selection of candidate genes and identification of families for linkage analysis is difficult. However, the advent of new technologies such as targeted exon capture and pyrosequencing have allowed us to put a twist on traditional gene identification methods and identify two excellent candidate genes we never would have selected with the tools and information we had available to us. The results of this thesis have also proven without careful investigation into family history it is difficult to differentiate between sporadic, familial and syndromic MD. In addition, genes such as *PCTK3* and *SLC45A3* which are responsible for familial MD may also be responsible for sporadic disease. MD was first described in 1861 and unfortunately the understanding of the disease has progressed slowly over the last hundred years. However, using multiple

genetic approaches in the last few years we have been able to identify three genes, *AQP4*, *PCTK3*, and *SLC45A3* which appear to be involved with the development of MD. The aims of this future grant could allow us to identify novel candidate genes for MD in an unbiased rapid manner and apply that information to both familial and sporadic disease. Since little is known about MD and its initiating factors, the identification of a genetic contribution to this disease may help to clarify disease pathogenesis and possibly lead to improved diagnosis and therapies.

APPENDIX A

LINKAGE AND ASSOCIATION STUDIES

Linkage Studies

A linkage study results in a genetic relationship between loci, whereas a genetic association is a statistical observation between alleles or phenotypes. Advantages of linkage studies are that the studies are unbiased when sufficiently large families are used. In addition, linkage can be used to identify a specific disease related gene. Unaffected family members are collected along with affected family members and used as controls. A weakness of linkage studies is the need for large families with multiple affected individuals, ideally three generations, 2 meioses to establish phase and 10 meioses to generate a significant LOD score. These large families can be especially difficult to obtain for adult onset disorders and complex diseases. Due to the numerous causes of adult onset hearing loss, tinnitus, and vertigo, recalling a family history of MD can be misleading, and there are very few large families that segregate the disease and are informative enough for a genetic study (Morrison, Mowbray et al. 1994), therefore very few linkage studies have been performed to date. Pitfalls of linkage analysis can include a false negative result following pooling of small families. In addition, including individuals that do not meet strict diagnostic criteria, or multiple susceptibility loci can lead to inaccurate linkage results (Morrison, Mowbray et al. 1994).

Association Studies

Association studies compare frequencies of specific alleles between a test population and a control population using either a candidate gene or a genome-wide association study design. An allele is associated with the disease in the test population if its frequency differs between cases and controls more than would be predicted by chance, provided the control population is representative of the test population and there is no ethnic substratification to produce false positive results (Morrison and Johnson 2002).

Variants are either selected and genotyped, usually single nucleotide polymorphisms (SNPs) or the entire gene interrogated by sequence analysis in a test and control population matched by gender, age, and ethnicity (Tabor, Risch et al. 2002). As a general rule, an association study is a powerful method to identify genetic components of a complex disease, and is appropriate for detecting common susceptibility alleles. Association studies can utilize unrelated singleton cases rather than large families. Weaknesses of association studies include the risk for population stratification (if disease frequency varies with ethnicity, a marker found at a high frequency in one ethnic group may have a positive association with the disease phenotype even if it is not the causative allele or near a causative allele) (Tsai, Choudhry et al. 2005). If a candidate gene study design is selected it presumes picking an appropriate candidate based on putative function, expression, and role in disease pathophysiology. Although association studies can identify a candidate region, it may be hard to narrow down the pathogenic variant (Cardon and Bell 2001). A genome-wide association study (GWAS), in contrast, is not hypothesis driven (Cardon and Bell 2001). The results of an association study can indicate an allele; in linkage disequilibrium with the mutant disease allele, linked to a founder mutation, causes a biologic susceptibility to disease, or be a false positive result (Morrison and Johnson 2002). When evaluating an association study one must note the study population size, if the controls are appropriately matched, the study design – candidate gene or GWA study, and the statistical analysis especially with regards to corrections for multiple allele testing. Association study statistics are highly influenced by sample size and studies of small cohorts should be interpreted cautiously. Presented in chronological order are the results of recent association studies for MD.

APPENDIX B

SELF REPORT MÉNIÈRE'S QUESTIONNAIRE

Ménière's Disease – A Molecular Genetic Study Self Report Questionnaire

Date: _________________ Date sample drawn: _____________

Sample type donated: (please circle appropriate response)

Blood Saliva Endolymphatic sac Skin

Contact Information:

Patient Name (Last, First, Middle): ______________________
Date of Birth: ______________
Patient Address: ____
Patient Phone Number: ____
Patient email: ____
Treating Physician: ____
Treating Physician Hospital: _______________________________________
Treating Physician Address: ____
Treating Physician Phone Number: __________________________________
Treating Physician Fax Number: ____________________________________

Personal Information:

Gender: Female Male

Current Age of Patient: ____

Race

Please circle one of the following:

Asian Black White Other ___________

Ethnicity

Please circle one of the following:

Hispanic Not Hispanic Other ___________

Date Patient diagnosed with hearing loss: _________________

Patient age at time of diagnosis: ____

Hospital or clinic where hearing loss was diagnosed? _____

How was it diagnosed? (i.e. audiogram, self-report) _______

Date Patient diagnosed with vertigo: ____________________

Patient age at time of diagnosis: ____

Length of vertigo attack: _________________________

Date Patient diagnosed with Ménière's disease: ______________

Patient age at time of diagnosis: _______

Diagnosing Physician: ___________________________

Have you ever received treatments for Ménière's disease?

Yes No

If yes, with what treatments? _________

Has anyone else in your family ever experienced poor balance, clumsiness, or dizziness?

Yes No

If yes, who? ____________________________________

If yes, what age? ______________

Do you have any blood relatives with hearing loss?

Yes No

If yes, how are they related to you? (i.e. maternal grandmother, etc)

Do you have any blood relatives that have been diagnosed with Ménière's disease?

Yes No

If yes, how are they related to you? (i.e. maternal grandmother, etc)

Have you or anyone in your family ever been diagnosed with migraine headaches?

Yes No

If yes, who? _________

If yes, what age? _________

Have you ever been treated for migraine headaches?

Yes No

If yes, with what treatments? _________

Have you or anyone in your family ever been diagnosed with glaucoma?

Yes No

If yes, who? _________

If yes, what age? _________

If you do not have Ménière's disease or Ménière's disease symptoms, what is your relationship to the patient with Ménière's disease?

____

Other relevant medical history:

Please mail or fax relevant medical records to:

Colleen Campbell
c/o Jodi Klein
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APPENDIX C

MATERIALS AND METHODS

Affymetrix GeneChip® Mapping 50K SNP Genotyping

Briefly, each sample was whole-genome amplified, fragmented, precipitated and resuspended in appropriate hybridization buffer. Denatured samples were then hybridized on prepared Affymetrix GeneChip® Human Mapping 50K Array Xba 240 for a minimum of 16 hours at 48°C. Following hybridization, the arrays were processed for the single base extension reaction, stained and scanned by a GeneChip Scanner 3000 7G (Affymetrix). Normalized bead intensity data obtained for each sample was loaded into the GeneChip Operating Software (GCOS) and GeneChip DNA Analysis Software (GDAS). The Affymetrix GeneChip® Human Mapping 50K Array Xba 240 uses >50,000 on a single chip with an average marker distance of 26 kb.

DNA was diluted to 50 ng/µL in reduced EDTA TE buffer (0.1 mM EDTA, 10mM Tris HCl, pH 8.0). 5 µL (50 ng/ µL) of genomic DNA was aliquoted into each well of a 96-well plate for a total of 250 ng. For the digestion reaction, 0.5 µL Xba I (20 U/ µL), 2 µL BSA (10X (1 mg/mL)), 2 µL NE buffer 2 (10X), and 10.5 µL H_20 were mixed with 5 µL gDNA (50 ng/ µL), the plate was sealed, vortexed, spun briefly at 2000 rpm for 1 minute, and placed in a thermal cycler for 120 minutes at 37°C followed by 70°C for 20 minutes. For the ligation reaction, 1.25 µL Adaptor Xba (5 µM), 2.5 µL T4 DNA Ligase buffer (10X), 0.625 µL T4 DNA Ligase (400 U/ µL), and 0.625 µL H_20 were added to each digested DNA sample on ice. The plate was sealed, vortexed, spun at 2000 rpm for 1 minute, then placed in a thermal cycler for 120 minutes at 16°C, and then 70°C for 20 minutes. The reaction was diluted with 75 µL molecular biology – grade H_20. Next, the following PCR Master Mix was prepared on ice; 44 µL H_20 was added to 10 µL 10X Pfx Amplification buffer, 10 µL 10X PCR Enhancer, 2 µL 50 mM $MgSO_4$, 12 µL 2.5 mM each dNTP, 10 µL 10 µM PCR Primer, 2 µL 2.5 U/ µL Pfx Polymerase.

For amplification, 10 µL of each diluted ligation sample was aliquoted into three separate wells of a new PCR plate, and 90 µL of PCR Master Mix was then added to each well. The plate was sealed, vortexed, and briefly spun. The following PCR program was used: 1 cycle of 94°C for 3 minutes, 30 cycles of 94°C for 15 seconds, 60°C for 30 seconds, and 68°C for 60 seconds, 1 cycle of 68°C for 7 minutes. Next, 3 µL of PCR product was then run on a 2% E-Gel *48* for 10 minutes to check the size. For purification, the three reactions for one sample were combined into a single well in a MinElute 96 UF PCR Purification Plate and placed on a vacuum manifold until the wells were completely dry. Next, PCR products were washed three times each with 50 µL of molecular biology water until the wells were completely dry. The MinElute plate was removed from the vacuum manifold gently tapped to remove an excess liquid from the bottom of the plate, and 40 µL EB buffer was added to each well. The plate was sealed and placed on a jitterbug shaker at room temperature for 5 minutes. The purified PCR product was then removed from the MinElute plate. The concentration of the purified PCR product was determined by UV spectrophotometry. For fragmentation, samples were combined with 5 µL of 10X Fragmentation Buffer on ice and vortexed. The Fragmentation Reagent was diluted to 0.04 U/ µL with Fragmentation Buffer and Molecular Biology Water while on ice and then 5 µL was added to each sample and mixed. The plate was sealed, vortexed, and briefly spun before being placed in a thermal cycler for 35 minutes at 37°C, followed by 95°C for 15 minutes. The plate was removed from the thermal cycler and briefly spun. Upon completion of the reaction, 4 µL of the fragmentation product was run on a 4% E-gel for 22 minutes. For labeling, 19.5 µL of Labeling Mix was aliquoted into the fragmentation plate on ice, the plate was sealed, vortexed, and briefly spun. The plate was placed in a thermal cycler at 37°C for 120 minutes, followed by 95°C for 15 minutes. Then, 190 µL of Hybridization Cocktail Master Mix was added to each DNA sample and denaturation completed at 95°C for 10 minutes followed by cooling on ice for 10 seconds. Samples were briefly spun then placed for 2 minutes at 48°C before being

injected into the array. Arrays were then allowed to hybridize for 16-18 hours at 60 rpm and 48°C. Next, the hybridization cocktail was removed from the array and replaced with 250 µL of Array Holding Buffer. Arrays were then washed and stained in a Fluidics Station 450 (Affymetrix), and scanned by a GeneChip Scanner 3000 7G (Affymetrix) according to manufacturer's instructions. Reference Genomic DNA, 103 (Affymetrix) and water were used as positive and negative controls, respectively. GeneChip Operating Software (GCOS) and GeneChip DNA Analysis Software (GDAS) were used to calculate cell intensity and scaling. (Gunderson, Steemers et al. 2006)

Short Tandem Repeat Polymorphic (STRP) Marker Confirmation

STRP markers were selected for each candidate interval with a LOD >1.0 and amplified for the entire family to reconstruct haplotypes. GeneMapper v4.0 was used to assign alleles. Haplotypes were manually reconstructed. A polymerase chain reaction (PCR) with10x Buffer PE Gold, $MgCl_2$ PE Gold (25mM), Amplitaq Gold, dNTPs, forward and reverse primers with fluorescently labeled 6-FAM-M13 labeled forward primer (5'-CACGACGTTGTAAAACGAC-3'), and ddH_2O was performed. To control for any run differences, the CEPH (Centre d'Etude du Polymorphisme Humain) control 1347-02 was included on each PCR plate. PCR products were then run on a 3130 XL Genetic Analyzer (AB) with a 50cm array and POP7 (AB). GeneScan500-LIZ (AB) was used as a size standard. GeneMapper v4.0 was used to assign alleles. Haplotypes were manually reconstructed.

NimbleGen Targeted Capture

The linked interval on chromosome 1q32.1-1q32.3 spanned chr1: 203523961-211830820 (8,306 kb). All known and hypothetical exons in this region (753 exons in 85 genes, 0.518Mb) were targeted for capture. Exons less than 50 base pairs (bp) that could not be merged with a neighboring exon were not included in the design. All exons were

padded with 30bp on the 5' end and 15bp on the 3' end to cover splice variants. Targets were brought up to a minimum size of 500bp. The targets were verified with SignalMap version 1.9 software from Roche NimbleGen, and the microarray was manufactured by Roche NimbleGen. The array design predicted 29-fold coverage for at least 50% of the bases, and 12-fold coverage for at least 90% of the requested bases.

A total of 2110 targets were requested covering 1,302 kb. Sequences were obtained from UCSC genome browser (hg18) (http://genome.ucsc.edu/). The human sequences for 753 exons (0.518Mb) in 85 known genes, alternative isoforms, as well as all ESTs were selected for the initial exonic design to cover the linked region on chromosome 1. The total amount of coding sequence was 1,208 kb, and 1,302 kb bases were requested to be placed on the array design of 2110 targets. The final design included 1959 targets covering 1,559 kb. Roche NimbleGen designed propriety non-unique probes to cover 89.7% of the primary targets requested. Roche NimbleGen was unable to include 160 kb (10.3%) in the array design, resulting in a total of 129 kb (8.3%) of coding sequence not included in the final design (<50bp exons and targets without probes). The array design predicted 29-fold coverage for at least 50% of the bases, and 12-fold coverage for at least 90% of the requested bases to facilitate detection of heterozygous SNPs. A 385K array was utilized (385,000 probes).

Briefly, four DNA samples were captured separately. Two from the proband (1020-15) obtained one year apart, one from his unaffected spouse (1020-16), and one from his affected son (1020-18). The design allowed for biological replicates of the proband as well as the segregation analysis within the family. Sequence capture and 454 pyrosequencing was performed at the 454 Life Sciences Service Center (454 Sequencing Center, 1 Commercial Street, Branford, CT 06405 USA, www.roche.com) using their modified protocol with 454 adaptors. Each captured DNA sample was placed in a gasket on one 454 GS FLX titanium chemistry run, expecting 30-55Mb sequence data per gasket.

Genomic DNA (gDNA) was extracted from the peripheral blood of the proband (MEN1020-15) and eluted in lo (1x) TE (reduced EDTA TE buffer (0.1 mM EDTA, 10mM Tris HCl, pH 8.0)). To confirm a single high molecular weight band gDNA was run on a 1% agarose gel. Nanodrop 7500 was used in triplicate to determine DNA concentration and quality. 5µg gDNA at a concentration of 500 ng/µL (A_{260}/A_{280} ratio ≥ 1.8; A_{260}/A_{230} ratio ≥1.9) was sent to the 454 Life Sciences Service Center (454 Sequencing Center, 1 Commercial Street, Branford, CT 06405 USA, www.roche.com) for capture, sequencing, data alignment, and preliminary analysis. Briefly, gDNA was fragmented and the single stranded DNA hybridized to the array. DNA not bound to the array was removed by washing, and the target enriched DNA was eluted from the array. Linkers were ligated to the DNA and then it was amplified by linker-mediated PCR. Probes for four loci conserved between human and mouse were included on the microarray for quality control analysis. Quantitative PCR (qPCR) for these loci was performed both prior to the array hybridization and following elution of the target DNA.

454 Pyrosequencing

Briefly, 5 µg of captured DNA in 100 µL of EB buffer was added to 70 µL of AMPure beads and incubated for 5 minutes at room temperature. The beads and DNA mixture was washed with 500 µL 70% EtOH twice, dried and the DNA eluted into 24 µL of EB buffer. The beads pelleted and the supernatant with the amplified DNA was transferred to a fresh tube, where it was nebulized, fragment end polished, and sstDNA library quality assessment and quantification is conducted.

Sanger Sequencing Variant Validation

PCR primers for candidate exons identified by sequence analysis were designed using Primer 3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). Amplimers containing candidate variants were amplified by a standard PCR protocol in all family members, and capillary sequencing of the exons performed with BigDye™ v3.1

Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA). The sequences were read by an ABI 3730s Sequencer (Perkin Elmer, Waltham, MA). Sequence variants segregating with MD in the family were analyzed *in silico* for potential functional consequences.

A 36bp deletion was visualized by running the PCR product of *SLC45A3* exon3-2 amplimer on a 2% agarose gel run at 80 volts for 5 hours (Figure 9).

APPENDIX D

SYNDROMIC MD

Ménière's disease and Migraine

First noted by Ménière and later by others, there is a higher prevalence of migraine and autoimmune disease in patients with MD than would be expected, both of which have associated genes lending support for a genetic predisposition to the development of MD (Brown 1941; Brown 1949; Morrison and Johnson 2002; Oliveira, Ferrari et al. 2002; Ruckenstein, Prasthoffer et al. 2002; Boyev 2005). Migraine is common and many patients with migraine have vestibular symptoms. Migraine-associated dizziness (MAD, migrainous vertigo) is when migraine directly causes vertigo attacks. MAD can make the diagnosis of MD difficult as both have overlapping symptoms, and are diagnoses of exclusion based on medical history as clinical diagnostic tests do not exist for either disorder (Minor 2005; Shepard 2006).

5% of MD patients in the U.S. were found to have migraine, and migraine was seen more frequently in MD patients than in the general Nigerian population (Wladislavosky-Waserman, Facer et al. 1984; Ibekwe, Fasunla et al. 2008). Patients with bilateral MD report migraine more than those with unilateral disease (Lopez-Escamez, Viciana et al. 2009). The expected lifetime prevalence of migraine and vertigo is 1.1%, however, the German National Health Survey reported the actual prevalence to be 3.2% (Lempert and Neuhauser 2009). Families segregating MD and migraine in an autosomal dominant manner have been reported and suggest a common genetic cause and a possible continuum between migraine and MD. Within a family, members may have either disorder, or both, and some report headaches prior to an MD attack or migraines only during adolescence and later development of MD. Cha and colleagues compared definite MD patients (n=50) and definite MD+ migraine (MMD) patients (n=18) (AAO-HNS 1995 and 2004 International Headache Society (IHS) criteria for migraine) and found

those with MMD were more likely to have an earlier age of onset, bilateral hearing loss, a family history of vertigo, and a family history of migraine than those with only MD (Cha, Kane et al. 2008). Cha and colleagues identified three sets of twins which all had migraine and were discordant for MD. This lead the authors to propose MD with migraine has high heritability with variable expression; the discordance seen in the two monozygotic twin pairs suggests environmental and epigenetic factors have a role in disease expression; partial MD is more common than definite MD if the two are caused by the same genetic factor; and a MD-migraine spectrum and common pathophysiology exist due to the presence of combinations of symptoms present in families (Cha, Kane et al. 2008). Interestingly, unless specifically asked, MD patients often do not mention other family members with MD or migraine symptoms (Oliveira, Bezerra et al. 1997). In our own experience, 25/101 (24.8%) of MD patients reported a personal of family history of migraine when questioned (unreported results).

If and how migraine may damage the ear and cause MD is controversial (Jen 2008). Headache or migraine prior to an MD attack has lead to the hypothesis that migraine could be a variable expression symptom of MD, and vascular changes in the central nervous system can result in a migraine and alter the microcirculation of the inner ear resulting in fluid imbalance, endolymphatic hydrops, and symptoms of MD (Oliveira, Bezerra et al. 1997; Oliveira, Ferrari et al. 2002; Cha, Brodsky et al. 2007). Alternatively, vasospasm of the small arteries of the inner ear may result in ischemic damage causing a susceptibility to develop MD (Cha, Kane et al. 2008). An altered ion channel present in the brain and ear may cause an increase in extracellular potassium resulting in both migraine and endolymphatic hydrops, and the additional potassium in the perilymph would be toxic to hair cells resulting in hearing loss (Ibekwe, Fasunla et al. 2008). A metabolic defect in the ear and brain has also been suggested to cause MD and migraine since most families segregating MD also have migraine (Jen 2008).

Ménière's Disease and Glaucoma

In 1949 Godtfredsen commented on the similarities between acute inflammatory glaucoma and MD as acute inflammatory glaucoma has increased aqueous humour and responds to diuretic treatment similar to MD. He then went on to report the occurrence of acute inflammatory glaucoma and MD in six patients which developed following trauma or infection (i.e. otitis media and iridocyclitis) (Godtfredsen 1949). In 1952 McGrath reported a case in which blunt force trauma induced glaucoma and several days later symptoms of MD, leading him to propose a common origin to MD and glaucoma exists (Godtfredsen 1949; McGrath 1952). However other studies have not noted the presence of glaucoma and idiopathic endolymphatic hydrops (fluctuating unilateral hearing loss, tinnitus, fullness of the ear and true vertigo) on exam (Berkowitz, Sessions et al. 1974). In our own experience, 13/101 (12.9%) of MD patients reported a personal of family history of glaucoma when questioned (unpublished results).

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