

**Mutational Analysis of a Candidate Gene Mapped to the Genetic
Locus for Hereditary Neuralgic Amyotrophy**

by

Tangee Davis

Departmental Honors Thesis

The University of Tennessee at Chattanooga

Biology

Project Director: Dr. Margaret Kovach

Examination Date: March 28, 2005

Dr. Deb Kreiss

Dr. Henry Spratt

Dr. H. Douglas Kutz

Examining Committee Signatures:

Project Director

Department Examiner

Department Examiner

Liaison, Departmental Honors Committee

Chairperson, University Departmental Honors Committee

ABSTRACT

Introduction: Hereditary Neuralgic Amyotrophy (HNA), also known as Brachial Plexus Neuropathy, is a rare hereditary disease that is characterized by recurrent episodes of severe arm and shoulder pain accompanied by muscle weakness, nerve deterioration, and sensory impairment of the nerves in the arm. Linkage studies have shown that HNA is an autosomal dominant disorder caused by a genetic defect localized to the long arm of the seventeenth chromosome between the 24th and 25th band (17q24-17q25). Physical characteristics accompanying the disease, such as hypotelorism (close-set eyes), short stature, cleft palate, and unusual skin folds around the neck and arms can be seen in some patients. Such genetic diseases have, in the past, been difficult to pinpoint or study due to the complexity of the genomic code. However, the completion of the human genome sequence by The Human Genome Project has made it possible to systematically screen genes localized to chromosome 17q24-q25 for mutations linked with HNA disease. As a result, many genes within this region have already been eliminated as candidate genes for HNA disease, however the causative defect for HNA remains elusive. The purpose of this experiment is to study a candidate gene for mutational defects that could be the causative agent of HNA.

Methodology: Blood samples were obtained from a four-generation HNA family (2 affected, 2 unaffected, and 1 obligate carrier). A candidate

gene for disease, Rhomboid or FLJ22341, was chosen for mutational analysis because its chromosomal location, tissue expression, and biochemical properties are consistent with the symptoms and genetics for HNA. PCR (polymerase chain reaction) analysis was used to target and amplify the coding exons of FLJ22341 from the genomic DNA of individual family members. Mutational analysis using the dideoxy method of DNA sequencing was performed on the Rhomboid gene, and the sequences were then analyzed for disease-associated mutations.

Results: Direct DNA sequencing identified two polymorphisms in FLJ22341; one of which was consistent with the pattern of disease. This cosegregating mutation was observed as an A→G transition in the heterozygous state in all affected individuals. The mutation localizes to the 5' UTR of the gene transcribed (151 bases upstream of the start codon) and is not likely to affect protein production; therefore, it was determined not to be the genetic defect responsible for HNA. A second polymorphism observed in only one individual represents a non-disease associated single nucleotide polymorphism (SNP). It is characterized as a T→C transition in the heterozygous state and is present at IVS9-75 (intervening sequence 75 bases upstream of exon 10). Its localization within intron 9 does not alter the splice-site consensus sequences and therefore, is not expected to affect transcript processing or protein expression.

Conclusion: HNA is a rare inherited neuropathic disorder displaying an autosomal dominant pattern of transmission. This study examined the genetic basis of HNA by screening the candidate gene FLJ22341 for disease-associated mutations. In FLJ22341, polymorphisms were identified; molecular evaluation determined they were not the cause of disease. Thus the gene responsible for HNA remains unknown and the search continues. Other genes in this same region such as FLJ34148 have yet to be examined and are excellent candidate genes of HNA disease due to their biochemical properties and expressivities. Future direction of this research will hopefully lead to the identification of the gene responsible for HNA and consequently bring about promising diagnostic tests and therapies.

ACKNOWLEDGEMENTS

I would like to thank Dr. Kovach for being my Departmental Honors Project Director and Dr. Spratt, Dr. Kreiss, and Dr. Kutz for being on my committee. I would like to thank Virginia Kimonis for clinical studies and HNA samples and Giles Watts for sequencing our samples. I would also like to thank the UHON faculty and staff for being so helpful and encouraging throughout this project as well as throughout my college career.

TABLE OF CONTENTS

| | |
|---|-----------|
| I. ABSTRACT..... | 2 |
| II. ACKNOWLEDGEMENTS..... | 5 |
| III. LIST OF FIGURES..... | 8 |
| IV. LIST OF TABLES..... | 9 |
| V. INTRODUCTION..... | 10 |
| A. Human Genome Project..... | 10 |
| B. Review of Hereditary Neuropathies..... | 13 |
| C. Hereditary Neuralgic Amyotrophy (HNA)..... | 15 |
| D. Genetic heterogeneity of HNA..... | 18 |
| E. Dysmorphic and Craniofacial Characteristics of HNA..... | 19 |
| F. Onset of HNA attacks..... | 22 |
| G. Physiological Evaluation of HNA..... | 23 |
| H. Treatment of HNA..... | 25 |
| I. Chromosomal Mapping of HNA Locus..... | 26 |
| J. Excluded Genes..... | 28 |
| K. Purpose of project..... | 31 |
| L. Candidate Genes..... | 31 |
| VI. MATERIALS AND METHODS..... | 34 |
| A. HNA Family Description..... | 35 |
| B. DNA Isolation and Locus Characterization..... | 37 |
| C. Selection of Candidate Genes..... | 38 |

| | |
|--|-----------|
| D. Primer Design..... | 39 |
| E. PCR Amplifications | 41 |
| F. Preparation of PCR Products for DNA Sequencing Reactions | 45 |
| G. DNA Sequencing/Analysis | 47 |
| VII. RESULTS | 50 |
| VIII. CONCLUSION | 56 |
| IX. REFERENCES | 59 |

LIST OF FIGURES

| | |
|---|----|
| 1. Figure 1: Brachial Plexus | 16 |
| 2. Figure 2: Dysmorphic characteristics present in HNA patients | 20 |
| A. Wrinkles on Neck | 20 |
| B. Wrinkles on arms of child | 20 |
| C. Wrinkles on scalp | 20 |
| 3. Figure 3: Facial Measurements | 22 |
| 4. Figure 4: Chromosome 17 | 27 |
| 5. Figure 5: Flow Chart of Methods | 34 |
| 6. Figure 6: Pedigree of samples | 37 |
| 7. Figure 7: Representation of FLJ22341 | 39 |
| 8. Figure 8: PCR Reaction | 44 |
| 9. Figure 9: Visualization of FLJ22341 exon-specific PCR products. | 46 |
| 10. Figure 10: Sanger Method of DNA Sequencing | 48 |
| 11. Figure 11: Chromatograms of FLJ22341 Polymorphisms | 51 |
| 12. Figure 12: Polymorphisms for FLJ22341 | 52 |
| 13. Figure 13: Post-transcriptional Splicing and Translation of FLJ22341. . | 53 |

LIST OF TABLES

| | |
|--|-----------|
| 1. Table 1: Excluded Genes within Critical Region for HNA. | 30 |
| 2. Table 2: Characteristics of Affected Individuals | 35 |
| 3. Table 3: Primer Sets for FLJ22341. | 40 |
| 4. Table 4: Polymorphism for FLJ22341. | 55 |

INTRODUCTION

Hereditary Neuralgic Amyotrophy (HNA), also known as Brachial Plexus Neuropathy, is a rare hereditary disease that is characterized by recurrent episodes of severe arm and shoulder pain accompanied by muscle weakness, nerve deterioration, and sensory impairment of the nerves in the arm. Studies have shown that HNA is an autosomal dominant disorder caused by a genetic defect localized to the long arm of the seventeenth chromosome between the 24th and 25th band (17q24-17q25). Diseases, inherited like HNA have, in the past, been difficult to characterize or study at the genetic level due to the complexity of the genomic code. However, the completion of the human genome sequence by The Human Genome Project has made it possible to systematically screen genes localized to chromosome 17q24-q25 for mutations linked with HNA disease.

Human Genome Project

The human genome, while the first vertebrate code sequenced, is also the most extensive (The International Human Genome Mapping Consortium, 2001a). It is a direct consequence of the Human Genome Project (HGP), which began in the early 1980's. By 1984, the idea of sequencing the human genome had been proposed (The International Human Genome Mapping Consortium, 2001a) and in 2001, the HGP along with a commercial organization called Celera simultaneously published the draft of the sequence,

which was then presented in the February 15, 2001 issues of Nature and Science respectively (The International Human Genome Mapping Consortium 409:860-915, 2001b; Venter et al., 291: 1304-1351,2001). The result of the adventurous undertaking has had profound medical and societal impact, most of which has yet to be realized. Previously, genetic research was much more difficult due to the fact that the genome is unpredictable; some chromosomes having many genes while others being very sparse (Pennisi, 2001). In fact, only 1-3% of the total genome actually encodes for genes that determine the expression of a protein. These gene-coding regions are transcribed or copied continuously in response to cellular needs. However, only certain portions of the transcript contain information for protein products. These areas are called exons (Baltimore, 2001). The remaining sequence consists of introns, which are spliced from the RNA transcript prior to protein production (Baltimore, 2001).

It is estimated that there are 30,000 to 40,000 protein-coding genes in the human sequence (Pennisi, 2001). Although this number is much smaller than expected, human genes are very complex and yield greater numbers of proteins through alternative splicing and exon shuffling (Pennisi, 2001). The actual number of genes is still debatable. Some genes are inactive and therefore difficult to discover or identify (Pennisi, 2001). Specific genes while only accounting for small portions of DNA, code for important biological functions (The International Human Genome Mapping Consortium, 2001a).

The hypothesized function of many genes is usually based on characteristics or properties that are shared with genes in other organisms. Genes such as these whose sequences are strikingly similar and predicted to have the same function are called orthologous groups (The International Human Genome Mapping Consortium, 2001a).

When analyzing sequences, changes within the code such as mutations or polymorphisms may be present. A mutation is an alteration from one specific hereditary condition to a different one (Griffiths et al., 2000). Mutations change the genetic code, but they are not always considered harmful (Baltimore, 2001). A polymorphism is any change in the nucleotide sequence and may be within coding or non-coding DNA. It represents a region of the code that differs from other individuals (Baltimore, 2001). If this base change is a single nucleotide, it can be referred to as a single nucleotide polymorphism (SNP). SNPs account for “most genetic variation between individual humans”, numbering over 1.4 million different ones in the human genomic code (Baltimore, 2001, pg.815; The International Human Genome Mapping Consortium, 2001). Mutations or polymorphisms can account for the causation of various genetic defects and diseases. The coding of the human genome has allowed for easier identification of human disease genes. Finding the specific gene will yield information such as disease symptoms, age onset, and hereditary transmission (Jimenez-Sanchez et al., 2001). The significance of this ability “will yield a deeper understanding of

disease and an enhanced integration of medicine with biology” (Jimenez-Sanchez et al., 2001, pg.853). The process of finding the causative agents for genetic diseases involves positional cloning. This includes “mapping the chromosomal region containing the gene by linkage analysis in affected families and then scouring the region to find the gene itself” (The International Human Genome Mapping Consortium, 2001a, pg.911). The HGP has helped with this by making information on candidate genes, mutational findings, and gene structure available (The International Human Genome Mapping Consortium, 2001a).

Review of Hereditary Neuropathies

Many phenotypes of hereditary neuropathies exist including chronic motor neuropathies, sensory neuropathies, painful neuropathies, acute generalized neuropathies, relapsing generalized polyneuropathies, and acute focal neuropathies (i.e. HNA). Differential diagnosis of a neuropathy that a person is affected by is based on family history, age of onset, disease course, inheritance pattern, electrophysiological examinations, and molecular analysis. Once the disease is diagnosed, a mode of disease management may be recommended (Pareyson, 2003).

Hereditary Neuropathy with liability to pressure palsies (HNPP) and HNA are both acute focal neuropathies genetically distinct but phenotypically similar and sometimes confused with each other. The brachial plexus can be

involved in HNPP, and pressure palsies can occur in HNA (Chance, 2001). HNPP is inherited in the autosomal dominant fashion with high penetrance, but it has inconsistent expression (Stögbauer et al., 2000). HNPP is a demyelinating neuropathy that has been linked to chromosome 17p11.2-12; specifically, it is caused by the deletion of the gene PMP22 (Chance, 2001). HNA is an axonal process pinpointed to 17q24-q25, but the gene responsible has still not been identified (Chance, 2001). HNA is distinguished from HNPP by the presence of dysmorphic and craniofacial features and pain in the affected areas (Ørstavik et al., 2001). However, these distinguishing features are not always part of the clinical presentation therefore differential diagnosis still remains challenging.

Another disease similar to HNA is Lumbrosacral Plexus Neurpathy (LSPN) (van Alfen and van Engelen, 1997). It affects the lumbrosacral plexus instead of the brachial plexus (van Alfen and van Engelen, 1997). Pain and atrophy occur in areas innervated by the lumbrosacral plexus such as the iliopsoas (hip flexors), quadriceps, adductors, and the gluteus muscles (van Alfen and van Engelen, 1997). Temporary paralysis of such areas as well as pain and weakness are common for LSPN (van Alfen and van Engelen, 1997). The disease usually is expressed either before the age of 20 or between 40 and 60 years of age (van Alfen and van Engelen, 1997). Most have complete recovery but still have some sort of residual affects (van Alfen and van Engelen, 1997). The lumbrosacral plexus may also be affected in HNA (Geiger

et al., 1974). LSPN and HNA are very similar in their expression and symptoms. Both, as of yet, are of unknown etiology. Pinpointing the cause of HNA may aid in finding the cause for LSPN. Clearly, gaining a better understanding of the genetic basis of these related disorders will contribute not only to better diagnosis but perhaps lead to more defined and effective treatments and therapies.

Hereditary Neuralgic Amyotrophy (HNA)

Hereditary Neuralgic Amyotrophy (HNA), also known as Brachial Plexus Neuropathy (BPN), is an autosomal dominant disorder that affects the brachial plexus nerve (Windebank, 1993). The brachial plexus is located in the shoulder region, and it extends from the lower cervical region to the axillary region (Gray, 1974). The joining of the anterior divisions of the 4 lower cervical and the greater part of the first dorsal nerves form the brachial plexus. It receives nerve bundles from the 4th cervical nerve and one from the 2nd dorsal nerve (Gray, 1974). Its branches extend through to the chest, shoulder, arm, forearm, and hand (Gray, 1974) (See figure 1).

Brachial Plexus

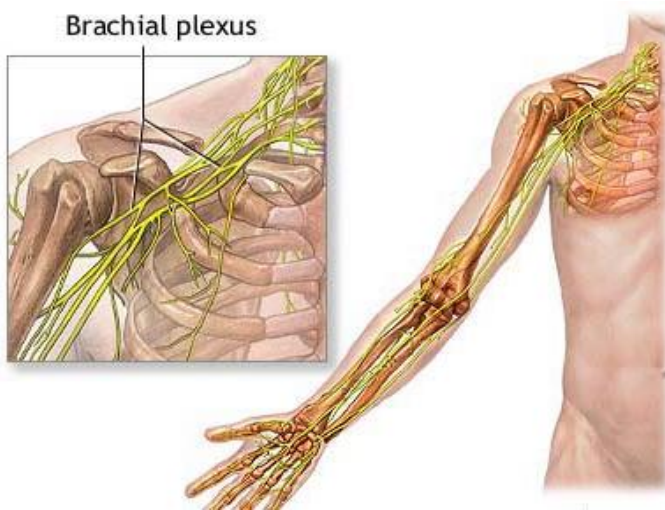


Figure 1: Location of the brachial plexus extending from the cervical region to the axillary region.

(AllRefer.com, 2003).

HNA has also been called Heredofamilial Mononeuritis Multiplex with Brachial Predilection (Taylor, 1960). This disease usually has a high penetrance of >90% meaning that over 90% of the people genetically disposed to HNA will present symptoms of disease (Kuhlenbaumer et al., 2000). The remaining 10% of individuals that are genetically predisposed to HNA, but do not present symptoms are called carriers. It is important to identify asymptomatic carriers so that the risk for offspring can be evaluated.

HNA is characterized by “acute brachial plexopathy with muscle weakness and atrophy, sometimes with sensory changes, preceded by severe muscle pain” (Pareyson, 2003). Episodes of pain in upper limbs that cause weakness and atrophy are usually seen (Windebank, 1993). While the pain can be anywhere from mild to sharp, it is usually severe, aching, burning pain and worsened when the affected limb is moved (Windebank, 1993).

The rapid onset of HNA is then followed by muscle weakness and/or paralysis (Tsairis et al., 1972). Regularly, the disease consists of several acute episodes with periods of recovery in between them that take place over a period of years (Windebank, 1993). After the attacks occur, weakness begins to set in and maximum weakness is present within a few weeks to a month; it will persist for several weeks to months (Windebank, 1993). The muscles become increasingly atrophied and fasciculation takes place (Windebank, 1993). Normally, weakness begins to emerge as the pain decreases, reaching its maximum level within two to three weeks following inception (Tsairis et al., 1972). Occasionally, paralysis of the vocal cords or aphonia can occur (Geiger et al., 1974). While sensory symptoms are possible, motor symptoms are more prominent (Meuleman et al., 2001). Some of the possible sensory symptoms may include vital hypaesthesia (impairment of tactile senses), dysaesthesia (unpleasant abnormal sensation), and paraesthesia (abnormal sensation) (van Alfen et al., 2000). Muscles innervated by the branches of the brachial plexus are usually affected. There is also a preference for the proximal limb muscles that are innervated by the upper trunk of the brachial plexus (Windebank, 1993). Occasionally, lower limbs and muscles innervated by cranial nerves may be included (Windebank, 1993). Most of the time only part of the plexus area is involved (Tsairis et al., 1972). The biochemical and molecular foundations of HNA are still unknown (Chance, 2001).

HNA occurs in 1.64 per every 100,000 individuals (Quan, 1994). In one clinical study of 99 patients, the male to female ratio was determined to be 2.4 to 1 (Tsairis et al., 1972). Of these affected individuals, 80% had sudden paralysis or paresis (slight or partial paralysis) (Tsairis et al., 1972). 70% of the cases had weakness in the first two weeks and 15% during the fourth week (Tsairis et al., 1972). Statistically, the brachial plexus that is affected varies. The study showed that 65 out of 99 patients had unilateral attacks; 35 were on the right and 30 were on the left. The person's individual dominance was not significantly correlated to these values (Tsairis et al., 1972). The remaining 34 patients had bilateral affects (Tsairis et al., 1972). 90% of the patients had abrupt severe pain (Tsairis et al., 1972).

Genetic heterogeneity of HNA

Two forms of HNA have been identified, one of which linked to chromosome 17q24-q25 (Watts et al., 2001). The two phenotypes are classic and chronic HNA. Classic HNA is characterized by relapsing, remitting symptoms, while the chronic kind is undulating and has persistent pain in between attacks (van Alfen et al., 2000). The classic form has been linked to 17q24-q25, while the chronic has not been chromosomally linked (Watts et al., 2001). Another distinguishing characteristic of the two forms of HNA is that no physical dysmorphisms are present with the chronic, non-linked version (Kuhlenbaumer et al., 2001).

Dysmorphic and Craniofacial Characteristics of HNA

Studies have shown that dysmorphic or physical characteristics can be present in many people affected by HNA; however, the features are not universally present (Windebank, 1993). The presence of dysmorphisms suggests that HNA may involve a “wider spectrum and involve nonneural tissue” (Jeannet et al., 2001). Possible features can include cleft palate, hypotelorism (close-set eyes), skinfolds, short stature, and narrow epicanthal folds (Jeannet et al., 2001). The skinfolds can be present on the arms of infants and toddlers, on the necks of women, and on the scalp (*cutis verticis gyrate*) (Jeannet et al., 2001). The arm and neck skinfolds have been found on more than 50% of affected women and on the forearms of several young affected children (Jeannet et al., 2001). These features may persist into adulthood in a milder form (Jeannet et al., 2001). Folds on the scalp, *cutis verticis gyrate*, are rare but were present in one particular case with a 19-year-old male who had already displayed one HNA attack when he was 4 years old. *Cutis verticis gyrate* is usually related to various encephalopathies, seizures, ocular abnormalities and various systematic illnesses. This patient, however, had not displayed any of these disorders, so the study concluded that *cutis verticis gyrate* was either related to the folds on the arms and neck or a rare coincidence (Jeannet et al., 2001). Figure 2 shows examples of wrinkles present on the arms, the neck, and the scalp.

Dysmorphic Characteristics Present in HNA Patients

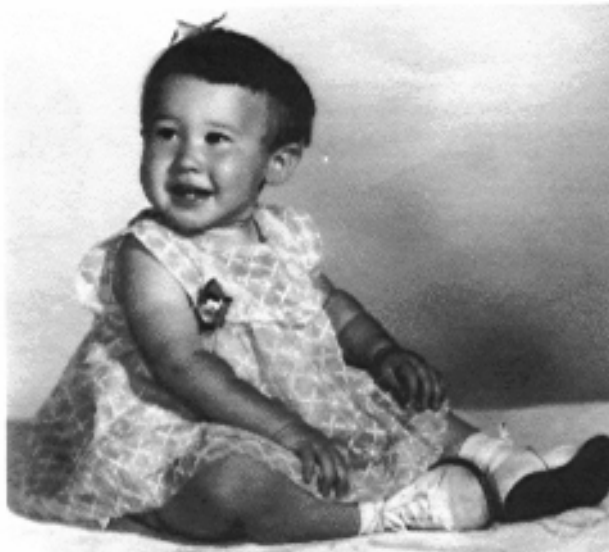


A.

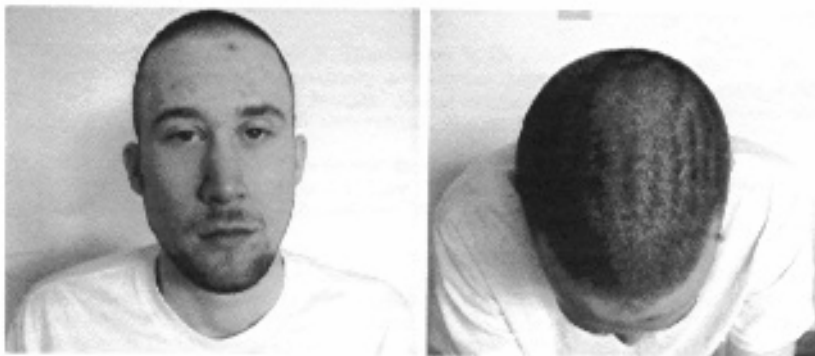
Figure 2: Physical dysmorphisms are found in many HNA patients (Jeannet et al., 2001).

A. shows hypotelorism and wrinkles around the neck. B. Shows a child with wrinkles around the arms. C. shows wrinkles on the scalp, cutis verticis gyrate.

All pictures show examples of hypotelorism.



B.



C.

One study established clinical evaluation based on medical exams, medical history, and facial measurements of controls and affected individuals to determine facial distributions for HNA dysmorphic features (Jeannet et al., 2001). Measurements that were taken were occipitofrontal circumference (OFC), intercanthal distance (ICD), interpupillary distance (IPD), and palpebral fissure length (PFL) (Jeannet et al., 2001). The interpupillary distance is the best measurement for the distance between the ocular globes and the best way to determine hypotelorism (Jeannet et al., 2001). See figure 3 for examples of ICD, IPD, and PFL. Results of the experiment showed reduced IPD, ICD, and PFL measurements in individuals with HNA compared to a control group. Height measurements were also decreased (Jeannet et al., 2001). A person with an IPD measurement that is 1 standard deviation below the average is said to have hypotelorism, and 67% of affected individuals fulfill this criteria (76% in this study) (Jeannet et al., 2001). The occurrence of cleft palate is not as numerous as other dysmorphic features, but it is still present in many HNA cases (Jeannet et al., 2001).

Facial Measurements

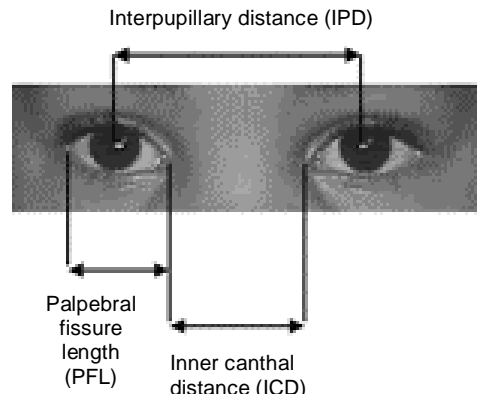


Figure 3: The palpebral fissure length is noticeably narrower in some patients with HNA. The inner canthal distance and the interpupillary are both commonly used to test for hypertelorism. (Jeannet, et al, Article)

The presence of dysmorphic and craniofacial characteristics in HNA patients suggests that HNA may have a pleiotropic affect, in that more than one phenotypic affect is observed as a result of a single genetic defect (Jeannet et al., 2001). HNA can also affect nonneural tissue such as connective or vascular tissue. Hypotelorism and cleft palate represent the possibility of abnormalities of a gene responsible for midline facial development (Jeannet et al., 2001). Since the causative agent of HNA is still unknown, it is unclear what factor relates it to these dysmorphisms (Kuhlenbaumer et al., 2000).

Onset of HNA attacks

HNA attacks can be triggered by certain causes such as physical, immunologic, and emotionally stressful aspects (Watts et al., 2001). Physical exertion or strenuous exercise of the affected limb has caused attacks to occur (Watts et al., 2001; Windebank, 1993). Sometimes, normal mechanical stress

can cause attacks in someone that has a natural predisposition for HNA (Windebank, 1993). Attacks have been caused by increased cold exposure of the affected limb (Kuhlenbaumer et al., 2000). Childbirth and surgery have triggered attacks of HNA, even if the activities do not directly involve the affected limb (Kuhlenbaumer et al., 2000; Watts et al., 2001). Immunologic events that trigger HNA include viral infections, immunizations, parturition (pregnancy), and puerperium (time between childbirth and when uterus returns to normal size) (Jeannet et al., 2001; Watts et al., 2001; Windebank, 1993). Problems occurring along side pregnancy suggest an immunological mechanism dealing with foreign acceptance (Windebank, 1993). No specific strain, trauma, or medications are associated with pregnancy that can cause an HNA attack (Windebank, 1993). These physical, emotional, and immunologic features can cause HNA attacks, but not all HNA patients are affected by them (van Alfen et al., 2000).

Physiological Evaluation of HNA

Examination of HNA patients is based in part on physiological evaluation testing of neurological function. Electromyography (EMG) and nerve biopsies are performed as part of a standard neuropathological examination. EMGs are representations of the electrical currents that are correlated to control of muscle movement (Stedman, 1995). Its main purpose is to distinguish muscular disorders from nervous disorders and to help

identify diseases (Rowland, 1995). EMG of affected limbs can reveal the acute stages of denervation and reduced interference pattern in muscles associated with the affected nerves (Kuhlenbaumer et al., 2000). Nerve biopsies are comprised of removing nervous tissue and examining it (Stedman, 1995). Nerve biopsy studies have shown axonal loss and demyelination in HNA cases (Meuleman et al., 2001a). Demyelination is when the myelin sheath, which covers the axon of neurons and is essential for appropriate nerve conduction, is lost or deteriorated (Stedman, 1995). Evidence of interruptions within the axon can also be seen at the brachial plexus of the affected limb and areas distal to the brachial plexus (Chance, 2001). Nerve conduction studies are used to help give vital information for diagnosing neuropathic disease (Rowland, 1995). Nerve conduction velocities (NCV) are either measured as normal or slightly reduced; normal results come from areas outside of the affected limbs, while being slightly slower in affected regions (Meuleman et al., 2001a). Another consequence of demyelination is reduced response of sensory neurons to stimuli in areas distal to affected nerves (Meuleman et al., 2001a). Demyelination and axonal loss can be responsible for the affects present in HNA. Without the necessary myelin on neurons, acetylcholine cannot be released, and muscle contraction cannot take place. These aspects can directly cause muscle weakness and atrophy. Other examinations that are performed but usually found to be negative with respect to HNA affection can include hematologic (white blood cell count elevation and red blood cell

sedimentation), biochemical, and cerebrospinal fluid tests (Windebank, 1993). Normal cerebrospinal fluid tests indicate that the affects of disease do not extend to the nerve roots (Windebank, 1993).

Treatment of HNA

While there is no definite treatment of HNA due to its unknown etiology, some types of treatment have been proven to help alleviate symptoms and help reduce weakness. Identification of the disease and distinguishing it from other neuropathic diseases is the first step to helping the affected patients (Windebank, 1993). Some individuals can be a carrier of the genetic defect without actually displaying symptoms of disease. One of the individuals included in this study is an example of an asymptomatic carrier (individual III:3). Carriers have a low degree of penetrance (Windebank, 1993). Sometimes, a brief course of steroids or intravenous methyl prednisolone can lessen pain and paresis, but this is not always successful (Windebank, 1993; Klein et al., 2002). Physical therapy of the affected limb is extremely important during recovery periods to maintain tissue and joint mobility (Windebank, 1993). Since HNA is triggered by many immunologic events, immune modulation may decrease or prevent attacks (Klein et al., 2002).

Chromosomal Mapping of HNA Locus

Through a series of genetic studies and haplotype analyses, the genetic localization of HNA has been refined to a 3.5 cM interval of chromosome 17q24-q25. It was originally narrowed down to the long arm of the 17th chromosome by genome-wide linkage studies using microsatellite markers spanning all 23 of the human chromosomes (Pellagrino et al., 1996). They showed significant linkage to the distal long arm of chromosome 17 (Lod score is greater than 3) (Pellagrino et al., 1996). After this it was linked to a 16 cM region to 4cM and most recently to 3.5cM. This region corresponds to the long arm of the 17th chromosome between bands 24 and 25 (see fig. 4) (Pellagrino et al., 1996; Pellegrino et al., 1997; Stögbauer et al., 1997; Meulman et al., 1999). Genetic linkage studies involve PCR-based DNA markers. The 3.5cM region is defined by molecular markers D17S785 and D17S802 (Meulman et al., 1999). These markers are short tandem repeats (STR) that are uniformly distributed throughout the genome and have been useful as guideposts in positional cloning and gene mapping studies (Watts et al., 2001; Kuhlenbaumer et al., 2000). Genetic linkage of HNA to these markers was determined using LOD score analysis. LOD scores reflect the probability of cosegregation of diseases and markers. A LOD score of 3 or higher is considered positive for linkage and corresponds to a 1/1000 likelihood of cosegregation of two genetic loci occurring by chance (Griffiths et al., 2000). In other words, with a LOD score >3 independent assortment of

the two loci is not statistically supported, indicating physical linkage of the two loci to the same chromosome. The closer the two loci, the less chance of crossing over between them (i.e., physical separation is low) and therefore, the higher LOD score.

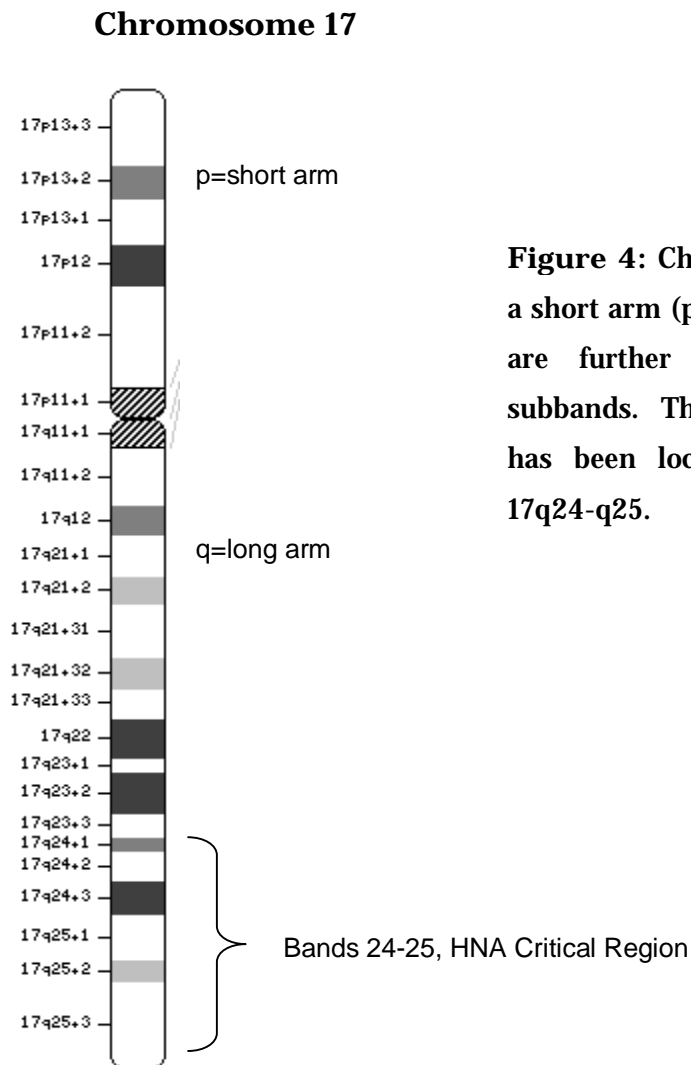


Figure 4: Chromosomes are split up into a short arm (p) and a long arm (q). These are further divided into bands and subbands. The gene responsible for HNA has been localized to a critical region 17q24-q25.

Excluded Genes

The search for the gene responsible for HNA has been ongoing for almost 22 years (Arts et al., 1983). Towards identifying the genetic cause of HNA, many genes within the critical region for HNA have been analyzed for mutations that could explain disease symptoms. Candidate genes for disease, that have subsequently been eliminated as causative agents are summarized in Table 1. All of these genes map to the critical region of HNA and have expression patterns consistent with disease pathology. ST6GALNAC2 encodes a putative sialyltransferase mapped to 17q25.1 and exhibits neural expression (Kuhlenbaeumer et al., 1998). Its importance to the immune system and nervous system development made it a good candidate for HNA causation (Kuhlenbaeumer et al., 1998). SFRS2 codes splicing factor including the *c-myc* ET-locus and is located at 17q24.1 (Kuhlenbaeumer et al., 1998). SFRS2 expresses transcripts that have only been detected in the thymus, which is one of the most important immune system organs (Kuhlenbaeumer et al., 1998). MSF is a MLL septin-like fusion protein that is associated with cyoskeletal filaments in cytoplasm and is located at 17q25.2-17q25.3 (Meuleman et al., 2001b). MSF can affect cell regulation and is expressed in the thymus, thereby a potential factor responsive to immunological insult (GeneReports, 2000c). TK1 is a thymidine kinase 1 soluble gene located at 17q25.3 and is expressed in lymph nodes, which are one of the primary sites for immune response (Meuleman et al., 2001b;

GeneReport, 2000d). ST6GalNAcI, a N-acetyl galactosamine-a 2,6-sialyltransferase 1 gene, can be expressed in dorsal root ganglia within the nervous system (Meuleman et al., 2001b; GeneReport, 2000e). SEC14L1's function is unknown, but is similar to SEC14 genes, which are a protein family that encode for cytosolic factors, suggesting a possible role in intracellular transport systems (Meuleman et al., 2001b; GeneReport, 2000f). SPHK1, which is located at 17q25.1, is expressed within the immune system and codes for an important kinase that affects cell growth and survival (Hünermund et al., 2004). TIMP2, a tissue inhibitor of metalloproteinase 2, plays an important role in the pathogenesis of experimental autoimmune neuritis by reducing and possibly preventing its occurrence (Hünermund et al., 2004). CYGB, located at 17q25.1 is a cytoglobin of unknown function, however its amino acid sequence is highly homologous to rat stellate cell activation associated protein gene (STAP) (Hünermund et al., 2004). STAP's expression in rats is accompanied by inflammatory cytokines that play an important role in the immune system when dealing with infection (Hünermund et al., 2004). The systematic examination and elimination of these candidate genes has resulted in a greater refinement of the HNA locus and will eventually lead to the identification of the gene for HNA.

Table 1: Excluded Genes within Critical Region for HNA

| <i>Gene Name/Accession Number</i> | <i>Function</i> | <i>Location</i> | <i>References</i> |
|-----------------------------------|--|-----------------|----------------------------|
| ST6GALNAC2/U14550 | <ul style="list-style-type: none"> • Putative sialyltransferase • important role in immune system and development of nervous system. | 17q25.1 | Kuhlenbaeumer et al., 1998 |
| SFRS2/M90104 | <ul style="list-style-type: none"> • Splicing Factor arginie/serine-rich 2 • includes <i>c-myb</i> ET-locus. | 17q24.1 | Kuhlenbaeumer et al., 1998 |
| MSF/NM_006640 | <ul style="list-style-type: none"> • MLL, septin-like fusion protein • associated with cytoskeletal filaments in cytoplasm. | 17q25.2-17q25.3 | Meuleman et al., 2001 |
| TK1/NM_003258 | <ul style="list-style-type: none"> • Thymidine kinase 1, soluble. | 17q25.3 | Meuleman et al., 2001 |
| ST6GalNAcI/Y11339 | <ul style="list-style-type: none"> • N-acetylgalactosamine-alpha 2,6-sialyltransferase I | 17q25.1 | Meuleman et al., 2001 |
| SEC14L1/NM_003003 | <ul style="list-style-type: none"> • Sec14-like 1 • unknown function. | 17q25.2 | Meuleman et al., 2001 |
| SPHK1/NM_021972 | <ul style="list-style-type: none"> • Sphingosine kinase 1 • product influences cell growth and survival. | 17q25.1 | Hünernmund et al., 2004 |
| TIMP2/NM_003255 | <ul style="list-style-type: none"> • Tissue inhibitor of metalloproteinase 2 • important role in pathogenesis of experimental autoimmune neuritis. | 17q25.3 | Hünernmund et al., 2004 |
| CYGB/NM_134268 | <ul style="list-style-type: none"> • Cytoglobin • unknown function . | 17q25.1 | Hünernmund et al., 2004 |

Purpose of project

This study proposes to continue the search for the gene responsible for HNA, through genetic analysis of additional candidate genes in a small family afflicted with HNA. Specifically, DNA was extracted from the 5 individuals in an HNA-affected family (2 affected, 1 obligate carrier, and 2 unaffected), and each sample was linked to the critical region for HNA on chromosome 17. A candidate gene was chosen due to its expression pattern and proposed function, and primers were designed for each coding exon of the candidate gene to be used in PCR amplifications. Following PCR amplification, the products were then prepared and sent off for DNA sequencing. The sequences were then analyzed for any disease-associated mutations that could be the causative agent of HNA.

Candidate Genes

Candidate genes were picked based upon their location, expression, and functions that could be compatible with the neurological and clinical phenotype of HNA. Genes associated with immune or hormone regulation might be good candidates because HNA is triggered by many immunologic activities (Klein et al., 2002). The causative gene does not have to be limited to the peripheral nervous system. Mutations could be associated with abnormalities in connective tissue or vascular tissue that supplies the brachial plexus (Pellegrino et al., 1997). The presence of dysmorphic characteristics

suggests that genes that participate in facial morphogenesis may be good candidate genes (Pellegrino et al., 1997).

The candidate gene being tested is called Rhomboid 4 or the genomic clone FLJ22341. This gene was chosen because its genomic localization, functional properties and tissue distribution were considered consistent with the phenotype of HNA. It is located within the critical region for HNA at 17q25.3, which means the long arm of the 17th chromosome, band 25, sub-band 3. FLJ22341 encodes for a Rhomboid-like protein (Koonin et al., 2003). The gene is expressed in many parts throughout the body including bone, brain, cartilage, muscular system, nervous system, peripheral nervous system, and many others (Bioinformatic Harvester, 2003). The gene functions as a critical part of signal transduction and controlling calcium levels (Koonin et al., 2003). The group of rhomboid proteins codes for intramembrane serine proteases, which contribute to the process of signal transduction (Koonin et al., 2003). Serine proteases are enzymes that break down proteins, and they contain serine and histidine residues that are associated with the active site of the catalysis; the function of serine proteases can affect immune responses (hyperdictionary, 2000). FLJ22341, also known as RHBDL6, is a rhomboid, veinlet-like protein-coding gene. Rhomboid is named as such because of its homology to the *Drosophila* rhomboid protein. It has been found in bacteria and eukaryotes, perhaps indicating a role in basic cellular function. (Bioinformatic Harvester, 2003). Rhomboid belongs to a group of important

integral proteins. The *Drosophila* homolog of rhomboid has been well characterized and has been shown to be responsible for the cleavage of a TGF- α -like growth factor that activates epidermal growth factor (EGF) receptors (Koonin et al., 2003). Rhomboid is expressed in tissues consistent with HNA pathology such as the peripheral nervous system and the muscular system. It also plays a part in immune response and muscle contraction through the regulation of signal transduction and calcium levels. All of these aspects of FLJ22341 make it a good candidate for HNA mutational analysis.

The DNA sequence of FLJ22341 was screened for genetic mutations that could possibly be responsible for the cause of the disease HNA. Molecular application used in the analysis includes the design and preparation of gene specific, oligonucleotide primers for use in polymerase chain reactions (PCR), DNA sequencing, gel electrophoresis, and purification of PCR products. The DNA samples of the individuals participating in the study have been coded to protect their identity. All that is known is their pedigree identification which includes gender, whether they are affected or not, and what relation they are to the other individuals in the pedigree.

MATERIALS AND METHODS

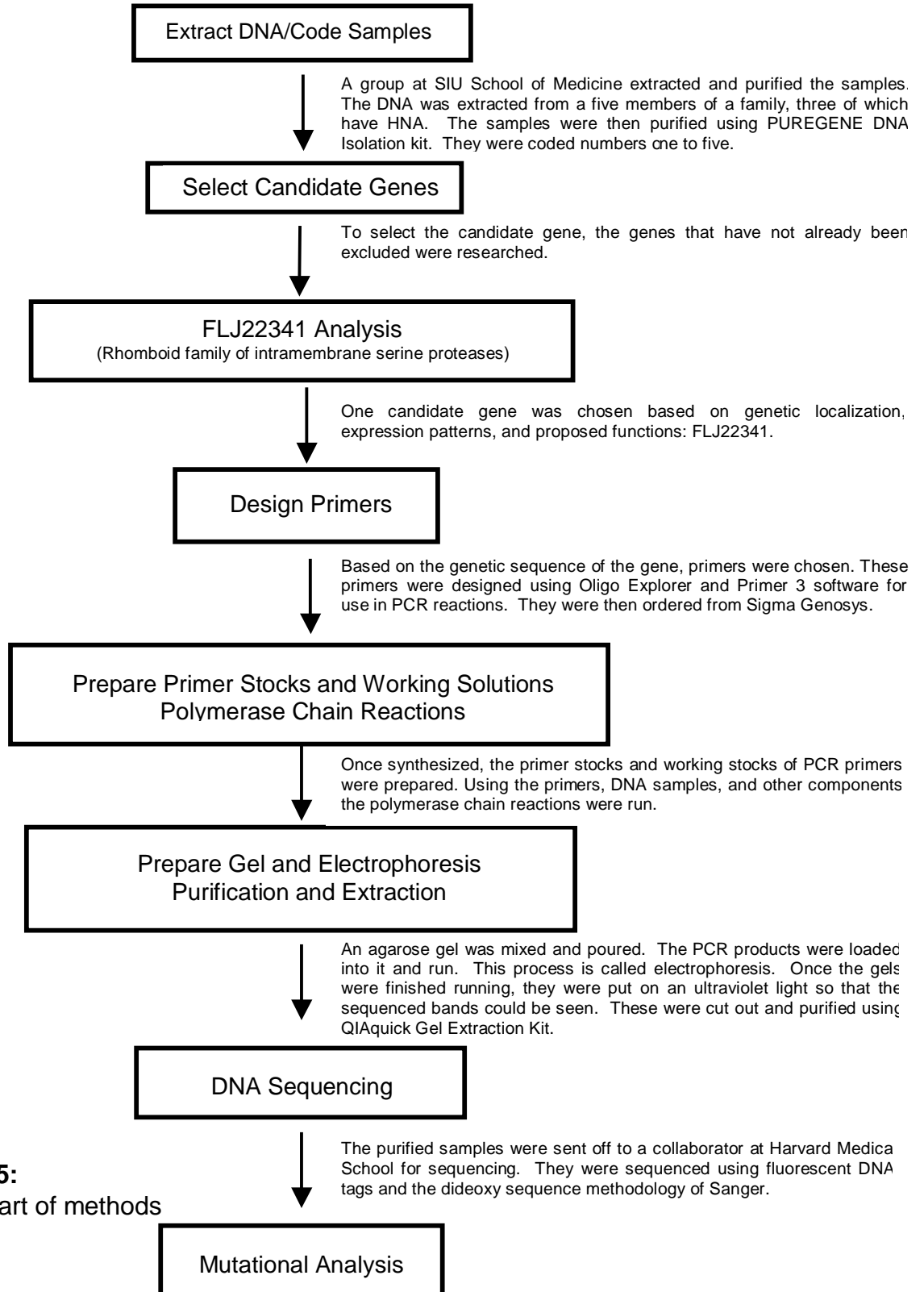


Figure 5:
Flow chart of methods

HNA Family Description

Members of a four-generation family presenting autosomal dominant HNA (Fig. 6) participated in this study after giving informed consent approved by the Springfield Committee for Research Involving Human Subjects. Clinical details are summarized in Table 2.

Table 2: Characteristics of Affected Individuals

| Pedigree No. | Age (Years) | Sex | Clinical Features |
|-------------------|-------------|-----|--|
| III:2 | 54 | M | <ul style="list-style-type: none">• Onset at age 17y.• Recurrent episodes of pain, weakness, and wasting of the muscles in his arms and hands. |
| III:3 | 52 | M | <ul style="list-style-type: none">• Asymptomatic• Some shoulder neuralgia (works as a painter)• Hypotelorism. |
| IV:5 (Proband) | 22 | M | <ul style="list-style-type: none">• Onset at age 10y.• Pain and weakness in arms with numbness in thumbs and fingers.• Atrophy and weakness of deltoid, suprascapular, rhomboid muscles, and abductor pollicis brevis.• Decreased reflexes. |

Molecular analysis was performed on five family members: two affected (III:2 and IV:5), two unaffected (III:4 and IV:2), and one obligate carrier (III:3). An obligate carrier is defined as an individual asymptomatic for disease but determined to have the genetic coding for it because the trait was passed on to their offspring. The proband (IV:5), defined as the individual who initially led to the testing of the family, is identified with an arrow on the pedigree (Fig. 6). The onset of HNA was noticed when he was 10 years old after a fall. He had pain in his upper right arm. The pain lasted

about two months. When he was 16, pain in his left forearm and biceps occurred and was soon followed by numbness in the thumb. When he underwent neurological examination, it was discovered that he had atrophy (weakening) of the deltoid (shoulder), suprascapular, rhomboid muscles, and abductor pollicis brevis. He also had reduced sensation and reflexes. Physical assessment showed hypotelorism and narrow palpebral fissures (the gap between the upper and lower eyelids) (see fig. 3). His father (III:3) had shoulder neuralgia but he attributed this to being a painter. Other than that, he was asymptomatic. Physically, he had evidence of hypotelorism and narrow palpebral fissures. The proband's (IV:5) uncle (III:2) has had episodes of pain in the arms since he was 17 years old. He also has weakness and atrophy of the muscles in his hands. II:2, the proband's (IV:5) paternal grandmother, had a history of paralysis in both arms since her pregnancy at the age of 24. She later developed leg weakness, which eventually contributed, to her death.

The family has a history of HNA occurrences as seen from the figure below. The proband is identified by IV:5 and sample number 5. His father, an obligate carrier, is III:3 and sample number 2. The uncle is sample number 1 and III:2. Samples 3 and 4 are unaffected family members. Sample 3 or III:4 is the proband's mother and sample 4 or IV:2 is the proband's sister.

Pedigree of Samples

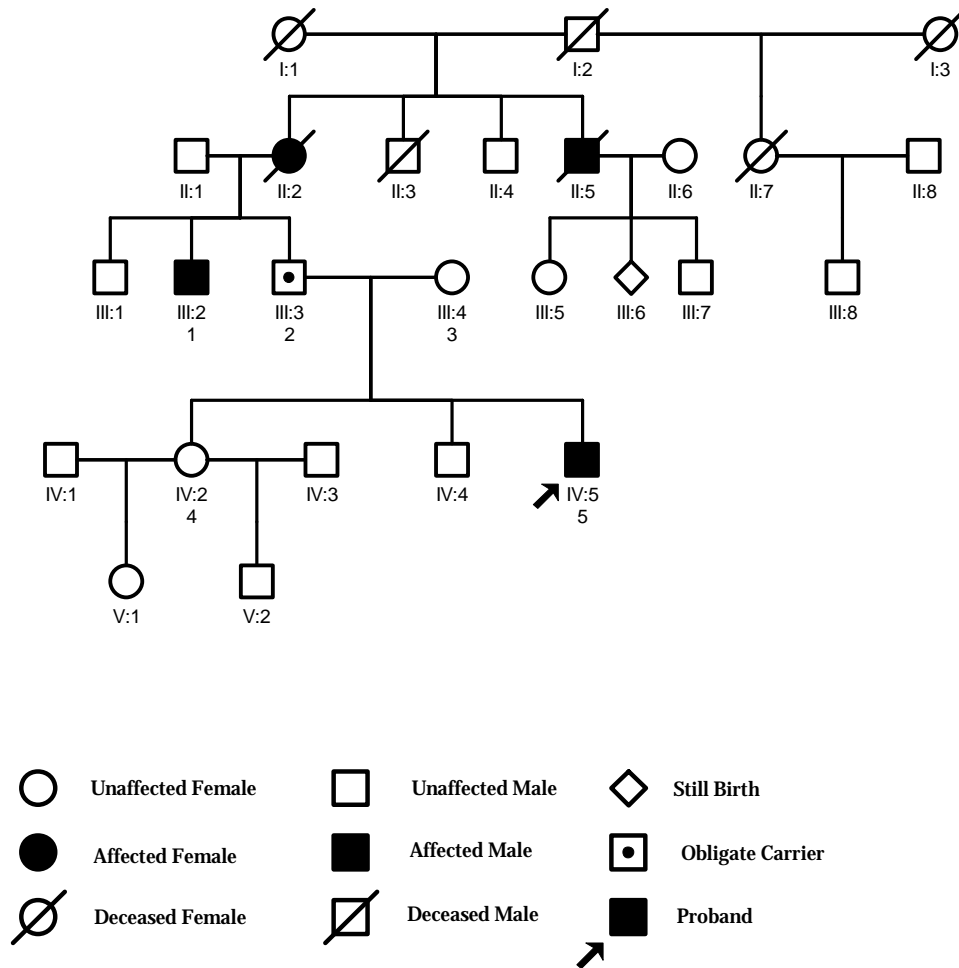


Figure 6: Pedigree of HNA family used in this experiment.

DNA Isolation and Locus Characterization

The genomic DNA was isolated and purified from blood samples using PUREGENE DNA Isolation Kit according to manufacturers instructions (Gentra Systems, 2005). Previous mapping studies on this family localized

the gene for HNA to a critical region on chromosome 17q24-q25 (Meulman et al., 1999). Specifically, the disease-associated region has been refined to a 1-4 Megabase interval on the long arm of chromosome 17, flanked by molecular markers DS1603 and DS802 (Meulman et al., 1999).

Selection of Candidate Genes

The candidate gene for the disease was chosen based on its biochemical properties and tissue expression pattern. Information on the genomic organization of the candidate gene was obtained from The Human Genome Browser (Human Genome Browser, 2004). Focus was given to genes that had cellular localization and activity that could potentially explain the HNA phenotype. The candidate chosen, FLJ22341, spans approximately 30,000 bases of genomic DNA and is comprised of nineteen exons, fourteen of which are coding. Figure 7 is a representation of genomic organization of FLJ22341. Another candidate gene, FLJ34148, extends approximately 96,000 bases and eighteen exons, seventeen of which are coding. This gene was originally meant to be included in the study, but its sequence is incomplete. For this study we were interested in identifying any mutations in the protein-coding portion of the gene, therefore the start and stop codons were localized, thereby defining the regions (exons) to be sequenced. The start codon (ATG) and the stop codon (TGA) were found that define a genomic region

encompassing exons 6 through 19 in FLJ22341 (see fig. 7). All the exons within this region are considered the coding regions.

Representation of FLJ22341

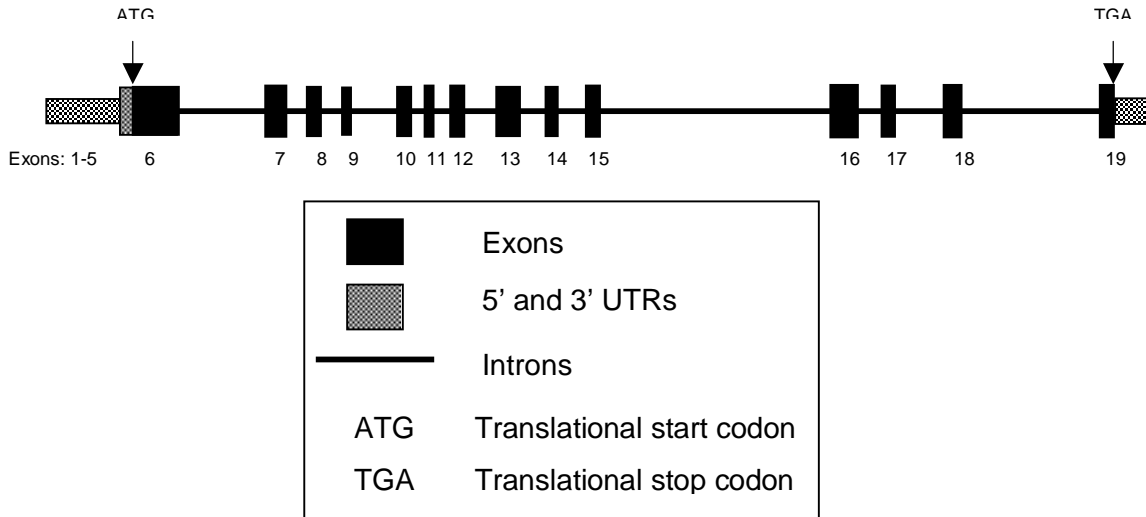


Figure 7: Representation of gene FLJ22341.

Primer Design

Polymerase Chain Reactions (PCR) were utilized to target and synthesize the coding exons of the candidate genes. These PCR products were then used as template material for DNA sequencing reactions. A schematic of PCR amplification is provided in figure 8. Primers for the PCR reactions were designed using the *Explorer* and *Analyzer* programs of the molecular software package *Oligo* (Oligo, 2003). Primer specifications were as follows: primer length—18-22 base pairs, melting temperature—55-65°C, and PCR product 200-600 bases. Based on these parameters, a forward and a reverse

primer pair flanking the genomic target site were designed specific for each coding exon. In some cases multiple exons were in close enough proximity, such that multiple exons could be amplified by a single set of primers (i.e. product length between flanking primers was ≤ 600 base pairs). Some exons, on the other hand were so large that more than one primer set was needed to cover the full exon. Designed primers were ordered from Sigma Genosys (Sigma Genosys, 2005). See table 3 for primers.

Table 3: Primer Sets for FLJ22341

| Primer Name | Sequence 5' to 3' | PCR product length | Exons Covered | T _m used in PCR reaction |
|--------------|----------------------|--------------------|--------------------|-------------------------------------|
| EX6FP | GGGATCCATGGGGAAAGGAG | 338 | 6 | 64°C |
| EX6RP | GAAGGGAGGGGCAAGGAAG | | | |
| EX7FP | GATGGGCTTGATGATTCC | 364 | 7 | 55°C |
| EX7RP | AGCAGCTCCACATAGGAC | | | |
| EX8/9FP | CCGGGAAAGTCACCATTG | 746 | 8 and 9 | 55°C |
| EX8/9RP | TCCTGATCCGCAGAAGAT | | | |
| EX10/11FP | GGCTCGTCTTAAAAGTTCC | 608 | 10 and 11 | 55°C |
| EX10/11RP | ACTCCTGGCTCAGATGATG | | | |
| EX12/13FP | GGGCAGAGGTGGAGTGATG | 707 | 12 and 13 | 61°C |
| EX12/13RP | GGAGACAGGGTTCAGATTG | | | |
| EX14/15FP | ACGTGGGTAGGTGGACGTG | 389 | 14 and 15 | 63°C |
| EX14/15RP | CAGAGGGCCCACAGGAAG | | | |
| EX16/17/18FP | AGGTGGGCAGTGGTTATTCG | 767 | 16, 17, and 18 | 63°C |
| EX16/17/18RP | GGGTGGAATAGGAGCAGCAG | | | |
| EX19aFP | ACCCACCTGATGCCTGAAG | 689 | 19 (first section) | 63°C |
| EX19aRP | GCTTGGGTCAGGATCTCACA | | | |

PCR Amplifications

Working solutions of each primer set were prepared in diH₂O (deionized water) at a final concentration of 20pmol/μl. A PCR reaction was set up for each primer pair as described in Table 3. The reagents for the PCR reaction were ordered from PE Biosystems (Applied Biosystems, 2005). A standard polymerase chain reaction consists of 1x PCR Buffer (50 mM KCl, 1.5 mM MgCl₂, 10mM Tris-HCl, pH 8.3), 0.8 μM of each forwards and reverse primer, 200 μM dNTPs and 2.5 units of Taq DNA polymerase. The Taq is a polymerase from a thermophilic bacterium called *Thermus aquaticus*. It catalyzes the synthesis of target DNA from the primers during the amplification process (Griffiths et al., 2000). The buffer provides salts and cofactors necessary for Taq polymerase activity while the dNTPs (deoxynucleotide triphosphates) provide the base unit of the synthesized DNA molecule to be used in the synthesis. To make laboratory work more manageable, master mixes were used such that a single, up-scaled reaction mix could be used for PCR amplification of all 5 DNA samples.

The standard guidelines for each PCR reaction as well as a master mix for five samples is seen below:

| <u>PCR Reaction (total 25 μl)</u> | <u>Master Mix (5.5x)</u> |
|--|---|
| 2 μ l of genomic DNA | (2 μ l genomic DNA added to individual PCR tubes) |
| 2.5 μ l of 10x buffer | 13.75 μ l 10 x buffer |
| 1.5 μ l 25 mm MgCl ₂ | 8.25 μ l MgCl ₂ |
| 2 μ l 100 mm dNTP mix | 11 μ l dNTP mix |
| 2 μ l 20 pmol/ μ l forwards primer | 11 μ l forward primer |
| 2 μ l 20 pmol/ μ l reverse primer | 11 μ l reverse primer |
| 0.2 μ l 12.5 units/ml Taq | 1.1 μ l taq |
| 12.8 μ l diH ₂ O | 70.4 μ l diH ₂ O |

All reactions were prepared on ice in special thin-walled PCR tubes that facilitate efficient temperature transfer throughout all steps of the PCR cycle. One to two drops of mineral oil are added to the top of each test tube to prevent boiling over of reactants. The PCR proceeded for 34 cycles in a DNA thermal cycler (PCR machine) programmed to the following parameters.

The PCR cycle is as follows:

1. 95°C for 7 minutes
2. 95°C for 1 minute
3. 60°C for 1 minute (this temperature is variable and specific to each primer set; for T_m values, see table 3)
4. 72°C for 1 minute
5. Repeat steps 2-4 34 times.
6. 72°C for 1 minute
7. 4°C for indefinite hold

Steps one and two function in denaturing the DNA to provide single-stranded DNA templates for DNA synthesis. Step three is specific (T_m) for each primer set and is usually somewhere around 60°C (See Table 3 for values). This step allows primer annealing to the denatured DNA template. Step 4 is the DNA polymerization phase and occurs at 72°C, the optimal temperature for Taq DNA polymerase. The first few steps are repeated so that the DNA is exponentially amplified with each additional cycle of polymerization; each new copy serves as a template for more copies in each cycle. A final extension phase (step 6) is added to ensure the synthesis of all PCR products is complete. Step 7 refrigerates the PCR products until they can be transferred to the freezer.

Schematic of PCR Reaction

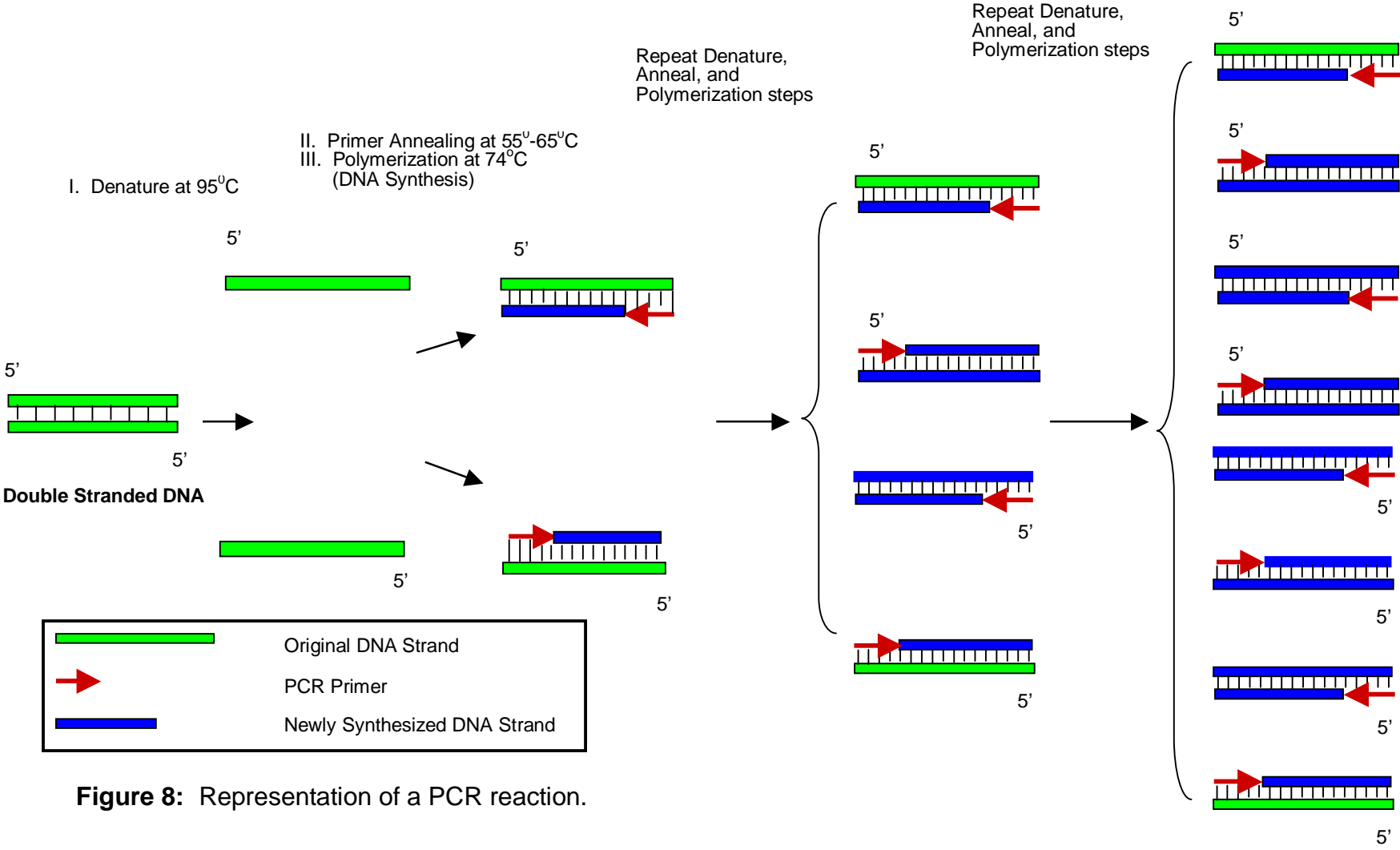


Figure 8: Representation of a PCR reaction.

Preparation of PCR Products for DNA Sequencing Reactions

Prior to DNA sequencing the PCR products were “cleaned up” from background genomic DNA and non-specific PCR products by a gel extraction method. Briefly, PCR products were separated according to size through a 1.5% agarose gel $\frac{1}{2}$ X TBE gel containing 0.5 $\mu\text{g/ml}$ EtBr (0.045 M Tris-borate, 0.001 M EDTA with Ethidium Bromide) (Sambrook et al., 1989). Ethidium Bromide is a nucleic acid specific dye that when bound to DNA and exposed to long wave UV light, emits a fluorescent signal thus allowing for visualization of DNA and PCR products. The agarose and TBE are heated until the agarose powder is completely melted, at which time it is poured into a gel form to solidify. Combs are placed in the top of the gel form to create wells that can be used for loading samples. Prior to loading samples into the wells, 6x-loading dye (30% glycerol in water, .25% bromophenol blue, 0.25% xylene cyanol FF) is mixed with each sample in a 1:5 ratio (Sambrook et al., 1989). The loading dye serves to give density to the sample and provides a color dye used in tracking electrophoresis. Tris borate EDTA buffer (TBE) was used in the electrophoresis. Each electrophoresis also contained a well loaded with a 100 base pair ladder marker that allows confirmation of PCR product length (New England Biolabs, 2005). After electrophoresis and size separation, the product bands can be viewed under UV trans-illumination (see fig. 9). Using a razor blade, each band containing desired PCR product is cut out and put in a separate test tube in preparation for purification.

Visualization of FLJ22341 exon-specific PCR products

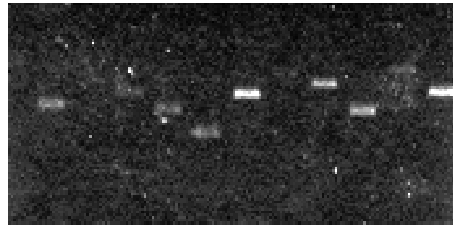


Figure 9: Visualization of FLJ22341 exon-specific PCR products
PCR products were subjected to agarose gel electrophoresis, stained with ethidium bromide and visualized by UV transillumination. Each PCR product band was cut out and purified in preparation for sequence analysis.

Purification of the extracted gel is done using the QIAGEN Gel Extraction and Purification Kit, according to manufacturers instructions (QIAGEN Inc., 2002). Each slice of gel is weighed to determine the correct amount of reagents to be used. Briefly, three volumes of Buffer QG (a chaotropic agent that facilitates solubility of gel by interrupting the ions in the water) are added per volume of gel. Dissolution of gel proceeds for 10 minutes at 50°C, or until gel has completely melted. One gel volume of isopropanol is added to each sample to increase the yield of DNA fragments <500 basepairs in length. QIA quick columns, which are made of a silica-gel membrane that is specialized for its binding properties are used to separate and purify the DNA from the samples (Qiagen, 2002). Upon application of samples the DNA infuses into the membrane when there are high levels of salt and optimal pH levels. Impurities pass directly through the column as flow-through (Qiagen, 2002). The DNA is bound to the column while wastes are separated and discarded. The column is washed sequentially with 500µl of

Buffer QG, followed by 750 μ l of PE buffer. At this point, the DNA is bound to the column matrix and is ready for elution with a low salt buffer (TE). The DNA is eluted from the column with 30 μ l TE. The sample is now ready for DNA sequence analysis.

DNA Sequencing/Analysis

All samples for DNA sequencing analysis were sent to a collaborator at Harvard Medical School. Each sample was prepared in PCR strip test tubes for automated sampling and analysis; each contained 15-30 ng DNA, 5 pmol primer (forward or reverse but not both). Fluorescently labeled dideoxy nucleotides are used in automated DNA sequence machines, such as the one used for this experiment. The machine allows for many samples to be simultaneously sequenced. Sequencing involved fluorescent DNA tags and the dideoxy sequence methodology of Sanger. Dideoxy nucleotides (ddNTPs) lack a 3'-hydroxyl group, which allows them to be incorporated into a growing chain of DNA and immediately terminate synthesis (Griffiths et al., 2000). By adding fluorescently labeled 4 ddNTPS (ddATP, ddTTP, ddCTP, or ddGTP) as well as regular deoxynucleotides (dNTPs) the sequencing method can proceed. Due to the dideoxy nucleotides integration into the growing strand at random points, a variety of truncated chain lengths that correspond to various points on the DNA chain will be created. The random process will create all possible fragments during synthesis, each one corresponding to the

locations of specific bases (Griffiths et al., 2000). The samples can be visualized by electrophoresis. With standard dideoxy sequence methodology, the gel has four lanes, one for each kind of base (Griffiths et al., 2000). When fluorescently labeled dideoxy nucleotides are used, only one lane is necessary because the dyes are different colors. Each fragment causes a band depending on its length, the shortest being on the bottom. The sequence can then be determined by reading up the gel as seen in figure 10 (Griffiths et al., 2000). Each ddNTP is tagged with fluorescent dyes, and a different color of dye is used for each base.

Sanger Method of DNA Sequencing

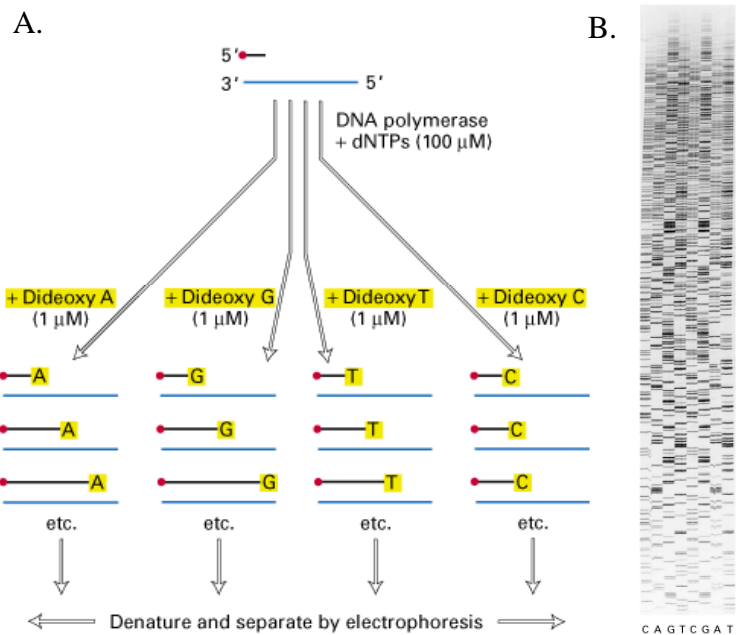


Figure 10: Sanger Method of DNA sequencing using dideoxy nucleotides. A. shows a schematic picture of how the ddNTPs terminate the sequence and producing fragments. B. shows an electrophoresed gel of the terminated fragments. From this, the sequence can be read. (Lodish et al., 2000).

After sequencing, each sequence output was evaluated for mutations or polymorphisms. The results of the sequencing are in the form of chromatograms represented by a color-coded sequence of bases. Individual sequences from each patient sample were aligned and compared in an exon-specific manner to determine any variations between affected and non-affected individuals. These could include but are not limited to: peak height, heterozygous features, deletions, insertions, and other mutations that occur in DNA. After they are compared, each polymorphism is analyzed to see if they lead to amino acid changes in the predicted protein sequence, which would possibly cause the disease HNA.

RESULTS

All participating individuals in this small family affected by HNA were subjected to sequence analysis of FLJ22341. Two polymorphisms were detected in the sequences (see fig. 12 and table 4). The first mutation was identified in affected individuals III:2, III:3, and IV:5 but not in unaffected individuals III:4 and IV:2, which makes it consistent with the pattern of disease. This polymorphism is characterized in exon 6 as an A → G transition found in the heterozygous state. It occurs at position 835 of the mRNA transcript. The transcript DNA sequence chromatogram for this polymorphism can be seen in figure 11. This polymorphic site of exon 6 is within the 5' UTR before the start codon (ATG). The 5' UTR (Untranslated Region) is a transcribed portion of the gene that does not get translated and whereas is not speculated to influence regulation of gene expression and mRNA stability. It is not part of the “coding” region of the gene and therefore is not predicted to affect protein structure and function. This heterozygous transition was confirmed through sequence analysis on the complementary strand of DNA.

Chromatograms of First Polymorphism

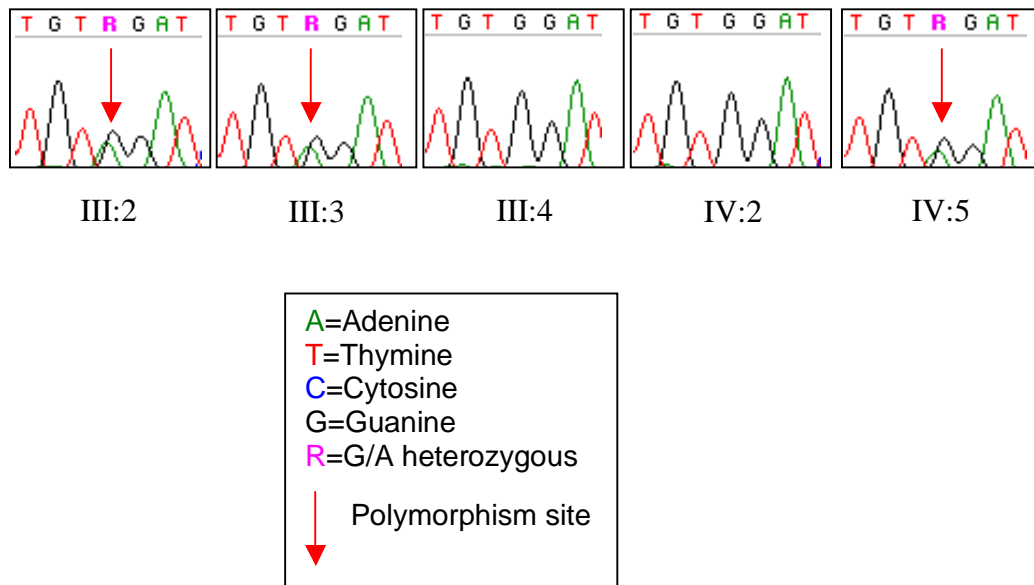


Figure 11: Chromatograms that show polymorphism characterized by an A \rightarrow G transition in the heterozygous state. This polymorphism is present in III:2, III:3, and IV:5. Notice an overlapping double peak (arrows) and a reduced “G-peak” height (black trace) in afflicted individuals (III:2, III:3, and IV:5) when compared to unaffected family members (III:4 and IV:2).

The second polymorphism identified was observed only in affected individual III:2, so it is not consistent with the pattern of disease. It represents a non-disease associated single nucleotide polymorphism (SNP). Characterized by a T \rightarrow C transition in the heterozygous state, it is located within the intron between coding exons 9 and 10, specifically IVS9-75 (intervening sequence 75 bases upstream of exon 10). The presence of this polymorphism was confirmed on a sequence of the complementary strand.

Since intron sequences are spliced out prior to translation of the protein, it is not predicted to alter the peptide sequence (see fig. 13).

Polymorphisms for FLJ22341

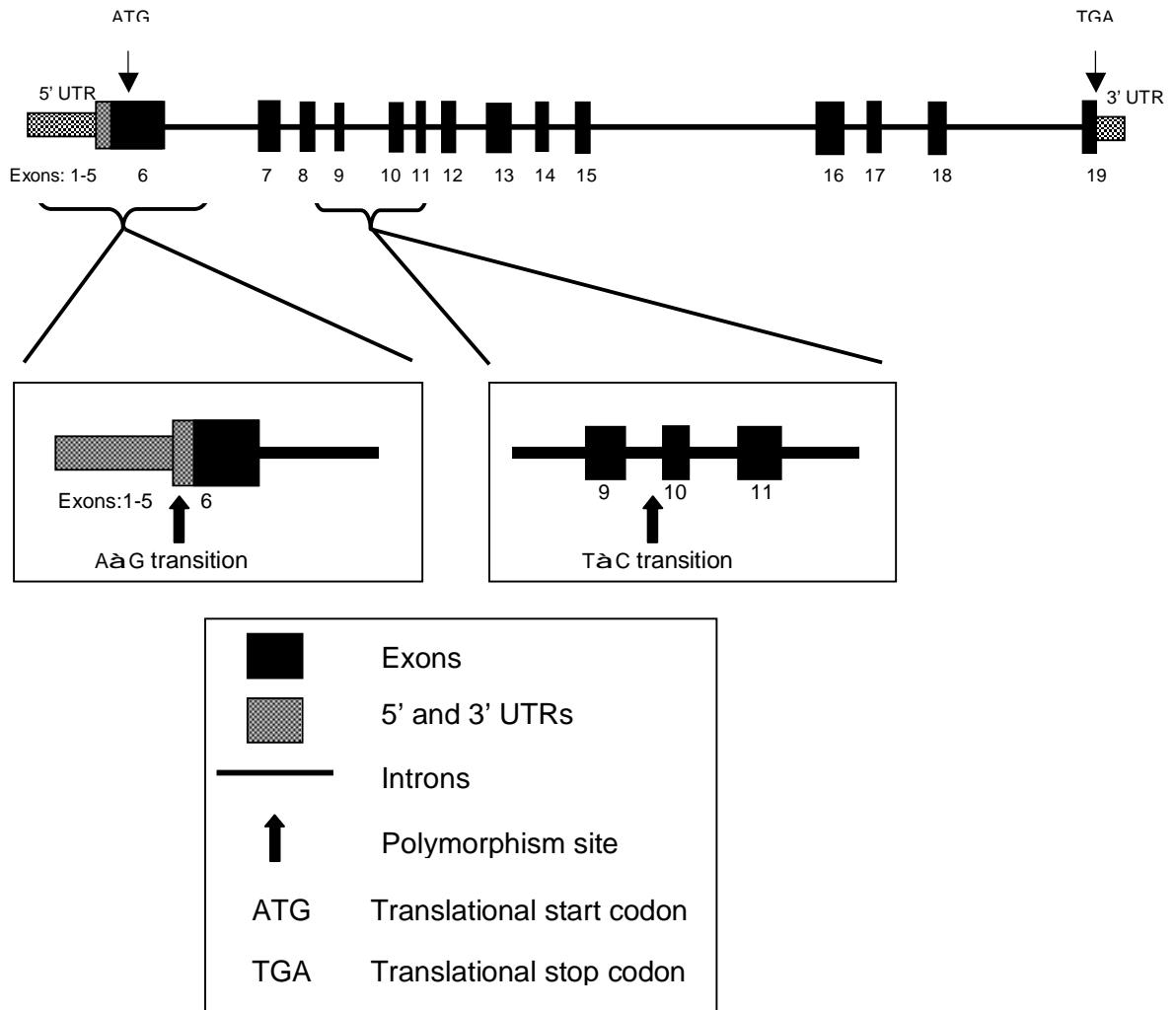


Figure 12: Representation of polymorphisms found for FJL22341 that shows where they are located on the gene.

Post-transcriptional Splicing and Translation of FLJ22341

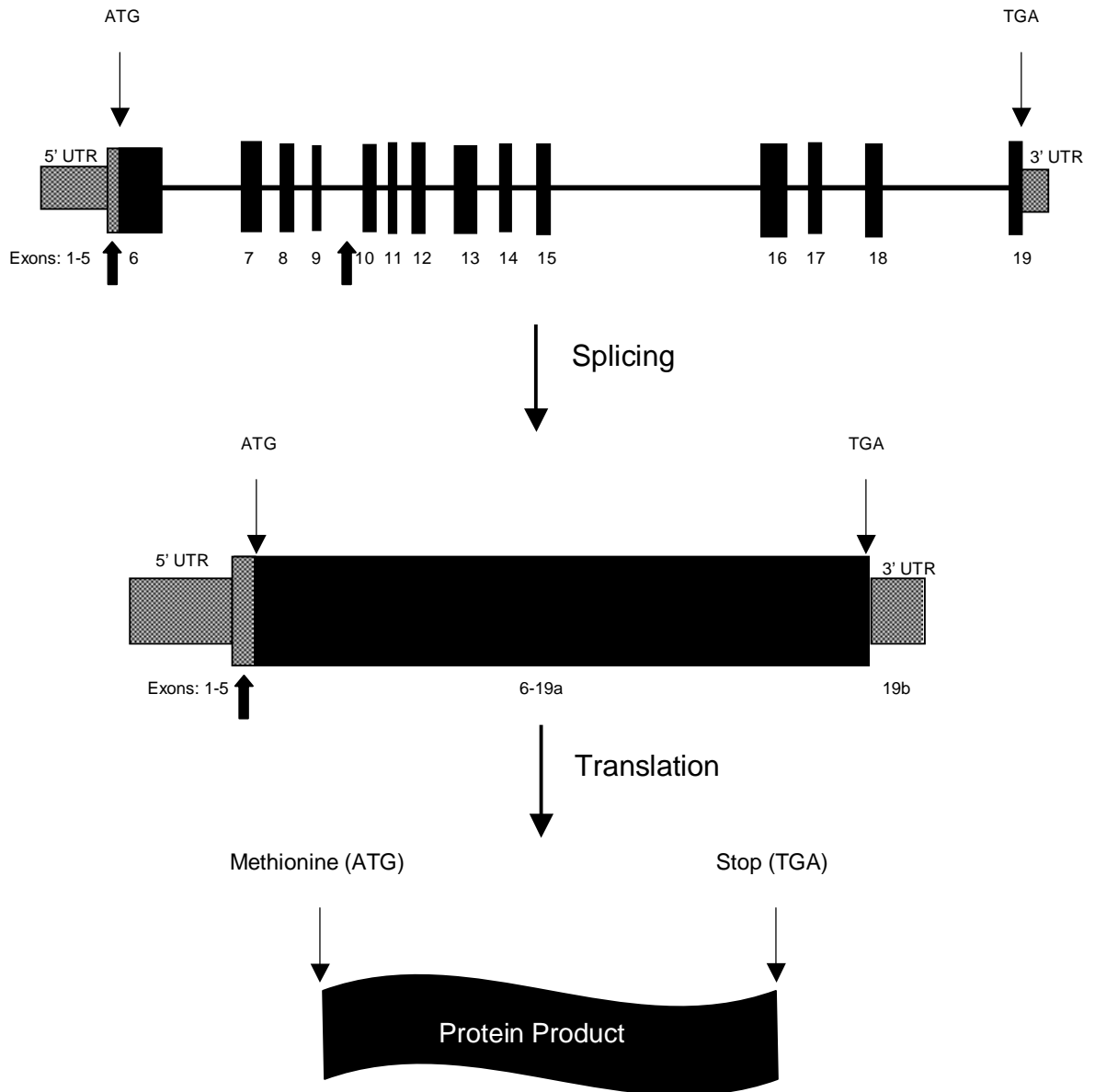
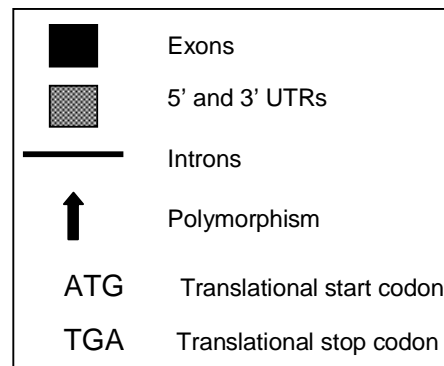


Figure 13: Notice that only the first polymorphism, the one located in the 5' UTR, is present after splicing.

Notice that in the protein product after translation, neither polymorphism is present.



Due to sequencing problems, one area of FLJ22341 spanning exons 12 and 13 has not been completely sequenced and analyzed for all individuals from this HNA family. Exon 12 accounts for 162 bases of coding sequence, and exon 13 is 110 bases. The PCR amplification reactions for exon 12/13 primers were inconsistent and unreliable. Quality PCR products for sequence analysis were obtained only for the samples of affected individuals (III:2, III:3, and IV:5). However, comparing the sequences from these affected individuals to the wild type [genotypical standard found in nature and used for laboratory stock (Griffiths et al., 2000)]. FLJ22341 sequence from the human genome browser database, it was determined that exon 12 and 13 did not contain any polymorphisms or disease associated mutations.

Table 4: Descriptions of Polymorphisms for FLJ22341

| Gene Name | Genbank Accession # | Number of Exons (Coding) | cDNA Length (bp) | Number of amino acids | Sequence variation and position* | Amino Acid Change | DbSNP [‡] accession # | Remarks |
|-----------|---------------------|--------------------------|------------------|-----------------------|---------------------------------------|-------------------|--------------------------------|--|
| FLJ22341 | NM_024599.2 | 19 (14) | 3,596 | 856 | c.835A>G Outside of cds; 5' UTR | None | None | <ul style="list-style-type: none"> • Consistent with disease pattern (found in III:2, III:3, and IV:5). • Not expected to affect splicing. |
| | | | | | IVS9-75T>C Within introns | None | None | <ul style="list-style-type: none"> • Only found in individual III:2 • Not expected to affect splicing. |

*Based on nomenclature defined by den Dunnen and Antonarakis, 2000.

‡DbSNP is the database of single nucleotide polymorphisms. It is a public-domain archive for many simple genetic polymorphisms.

CONCLUSION

HNA is a rare inherited neuropathic disorder displaying an autosomal dominant pattern of transmission. It has been linked to a 3.5cM interval on chromosome 17 (17q24-25). Based on gene expression patterns and biochemical properties a candidate gene for disease was chosen from this region for sequencing and mutational analysis. This study examined the genetic basis of HNA by screening the candidate gene FLJ22341 for disease-associated mutations. In FLJ22341, 2 polymorphisms were identified. The first polymorphism which was identified in all three affected samples (III:2, III:3, and IV:5), is characterized by substitution of guanine with adenine in the 5'UTR region of exon 6. Even though it is consistent with the pattern of disease, it is not predicted to affect mRNA splicing or alter the protein sequence because it is located prior to the translation start codon (see fig. 13). Therefore, this polymorphism is not implicated as the causative agent of HNA. The second polymorphism, which was only present in one individual (III:3), occurs within the intron between exons 9 and 10. Since introns are removed prior to translation this mutation will not have any effect on expression of the rhomboid protein and therefore is not expected to have a phenotypic effect (see fig. 13).

Due to difficulties in PCR amplification, exons 12 and 13 were not completely sequenced and analyzed for all family members. However, the reliable sequences from affected individuals were compared to the wild type of

FLJ22341's sequence from the Human Genome Browser to check for disease-associated mutations and polymorphisms. None were identified. Overall, affirming that FLJ22341 is not responsible for the causative agent for HNA. In summary, mutational analysis of FLJ22341 identified two polymorphisms in FLJ22341. Due to their location in non-coding regions and inconsistency with the pattern of disease, FLJ22341 was eliminated as a candidate gene of HNA. Thus the gene responsible for HNA remains unknown and the search continues.

Other genes within the critical region of disease are possible candidates for molecular analysis due to their expressivities and locations. FLJ34148, which is a transcript that is similar to the zinc finger class of transcription factors, is a prospective gene that seems likely due to its expressivity. Transcription factors are proteins that regulate the expression of other genes, and a defect in a transcription factor like FLJ34148 could explain the pleiotropic effect observed in HNA. This gene is also found within the critical region for HNA at 17q25.3. This can be expressed in skeletal muscle, prostatic epithelial cells, metastatic prostatic bone lesions, natural killer cell line, and many other places throughout the body. It is expressed in appropriate muscles and within the immune system, which makes it a good candidate for HNA causation. FLJ12878 or E2-230K, which is located at 17q25.1, is also a good candidate gene. It is an ortholog of mouse ubiquitin-conjugating enzymes and is expressed in the peripheral nervous system with high levels of

penetrance (GeneReport, 2000a). Another possible candidate, KIAA1582, is present at 17q25.3 and functions as a nuclear pore complex component that plays an important role in nuclear export and protein transport. It is expressed in bone tissue, lymphatic tissue, bone marrow, and dorsal root ganglia (GeneReport, 2000b).

This kind of genetic research is extremely important because discovering the causative gene would provide valuable information about the disease and how it affects the body. This beneficial information would aid in creating a treatment for the disease and possibly lead to finding causative agents for related disease such as Lumbrosacral Plexus Neurpathy (LSPN), which is related to HNA. It is expressed similarly, and sometimes symptoms overlap between these two diseases. Finding the mutations and polymorphisms that account for the causation of various genetic defects and diseases will yield information such as disease symptoms, age onset, and hereditary transmission (Jimenez-Sanchez et al., 2001). This kind of research is advantageous for future biological and medical improvements and developments: "It will reveal general principles of human disease" (Jimenez-Sanchez et al., 2001, pg.853). Increased knowledge of genetic diseases will allow for enhanced incorporation of biology with medicine (Jimenez-Sanchez et al., 2001).

REFERENCES

- (2003). Brachial plexus. AllRefer.com. Retrieved August 8, 2004:
<http://health.allrefer.com/health/brachial-plexus-brachial-plexus.html>.
- (2003). Bioinformatic Harvester. Retrieved March 1, 2005:
<http://harvester.embl.de/harvester/Q96E/Q96E02.htm>.
- (2003). Oligo: Molecular Biology Insights. Retrieved July 2004:
<http://oligo.net/>.
- (2005). Applied Biosystems. Retrieved March 14, 2005:
<http://www.appliedbiosystems.com/>.
- (2005). Gentra Systems. Retrieved March 2005: <http://gentra.com/>.
- (2005). New England Biolabs, Inc. Retrieved March 14, 2005:
<http://www.neb.com/nebecomm/default.asp>.
- (2005). Sigma Genosys. Retrieved March 14, 2005: <http://www.sigma-genosys.com/index.asp>.
- Arts, W.F., Busch, H.F., Van den Brand, H.J., Jennekens, F.G., Frants, R.R., and Stefanko, S.Z. (1983). "Hereditary neuralgic amyotrophy. Clinical, genetic, electrophysiological and histopathological studies." Journal of Neurological Science, 62(1-3):261-79.
- Baltimore, David. (2001). "Our genome unveiled". Nature 409(6822): 814-816.

- Chance, P.F. (2001). "Molecular basis of hereditary neuropathies." Physical medicine and rehabilitation clinics of North America, 12(2): 277-291.
- den Dunnen, J.T. and Antonarakis, S.E. (2000). "Mutation nomenclature extensions and suggestions to describe complex mutations: a discussion." Human Mutations. 15(1): 7-12.
- Geiger, Leonard R., Mancall, Elliott L., Penn, Audrey S., and Tucker, Samuel H. (1974). "Familial Neuralgic Amyotrophy: Report of three families with Review of the Literature." Brain, 97: 87-102.
- Genome Bioinformatics Group of UC Santa Cruz. (2004). Human Genome Browser Gateway. Retrieved January 2004: <http://genome.ucsc.edu/cgi-bin/hgGateway>.
- Gray, Henry. (1974). The Brachial Plexus. T. Pickering Pick and Robert Howden (Eds.), Gray's Anatomy. (pp. 764-76). Philadelphia: Running Press.
- Griffiths, Anthony J.K., Miller, Jeffrey H., Suzuki, David T., Lewontin, Richard C., and Gelbart, William M. (2000). An Introduction to Genetic Analysis. New York: W.H. Freeman.
- Hünermund, Gert, Schirmacher, Anja, Ringelstein, Bernd, Young, Peter, Watts, Giles D., Meuleman, Jan, et al. (2004). "Genomic Organization and Mutation Analysis of Three Candidate Genes for Hereditary Neuralgic Amyotrophy." Muscle and Nerve, 29: 601-604.

The International Human Genome Sequencing Consortium. (2001). "Initial sequencing and analysis of the human genome". Nature 409(6822): 860-915.

The International Human Genome Mapping Consortium. (2001). A physical map of the human genome Nature 409: 934 – 941.

Jeannet, P.Y., Watts, G.D., Bird, T. D., and Chance, P. F. (2001). "Craniofacial and Cutaneous Findings Expand the Phenotype of Hereditary Neuralgic Amyotrophy." Neurology, 57: 1963-8.

Jimenez-Sanchez, Gerardo, Childs, Barton and Valle, David. (2001). "Human disease genes". Nature 409(6822): 853-855.

Klein, C.J., Dyck, P.J.B., Friendenberg, S.M., Burns, T.M., Windebank, A.J., and Dyck, P.J. (2002). "Inflammation and neuropathic attacks in hereditary brachial plexus neuropathy." Journal of Neurology, Neurosurgery and Psychiatry, 73: 45-50.

Koonin, Eugene V., Makarova, Kira S., Rogozin, Igor B., Davidovic, Laetitia, Letellier, Marie-Claude, and Pellegrini, Luca. (2003). "The Rhomboids: a Nearly Ubiquitous Family of Intramembrane Serine Proteases that Probably Evolved by Multiple Ancient Horizontal Gene Transfers." Genome Biology, 4(3), R19. 28 Feb. 2003 <<http://www.pubmedcentral.nih.gov/articlerender.fcgi?tool=pubmed&pubmedid=12620104>>.

Kuhlenbaumer, G., Meuleman, J., Schirmacher, A., Stögbauer, F., Ringelstein, E.B., Wehnert, M., et al. (1998). "Mutation analysis of a putative sialyltransferase gene, the SFRS2 splicing factor gene and the *c-myb* ET-Locus in two families with hereditary neuralgic amyotrophy (HNA)." Ann. Human Genetics, 62: 397-400.

Kuhlenbaumer, G., Schirmacher, A., Meuleman, J., Tissir, F., Del-Favero, J., Stögbauer, F., et al. (1999). "A Sequence-Ready BAC/PAC Contig and Partial Transcript Map of Approximately 1.5 Mb in Human Chromosome 17q25 Comprising Multiple Disease Genes" Genomics, 62: 242-50.

Kuhlenbaumer, G., Stögbauer, F., Timmerman, V., and De Jonghe, P. (2000). "Diagnostic guidelines for hereditary neuralgic amyotrophy or heredofamilial neuritis with brachial plexus predilection." Neuromuscular Disorders, 10: 515-517.

Kuhlenbaumer, G., Meuleman, Jan, Jonghe, Peter De, Falck, Björn, Young, Peter, Hünermund, Gert, et al. (2001). "Hereditary Neuralgic Amyotrophy (HNA) is genetically heterogeneous." J. Neurol, 248: 861-865.

Lodish, Harvey, Berk, Arnold, Zipursky, Lawrence S., Matsudaira, Paul, Baltimore, David, and Darnell, James. (2000). Molecular Cell Biology. (4th ed.). New York City: W. H. FREEMAN and Company. Retrieved March 9, 2005, from http://departments.oxy.edu/biology/Stillman/bi221/092200/lecture_notes.htm.

Meulemann, J., Kuhlenbaumer, G., Schirmacher, A., Wehnert, M., De Jonghe, P., De Vriendt, E., et al. (1999). "Genetic refinement of the hereditary neuralgic amyotrophy (HNA) locus at chromosome 17q25." Human Genetics, 7(8): 920-7.

Meuleman, J., Kuhlenbäumer, G., Audenaert, D., Hünermund, G., Hor, H., Young, P., et al. (2001). "Mutation analysis of 4 candidate genes for hereditary neuralgic amyotrophy (HNA)." Human Genetics: 108, 390-393.

Meuleman, J., Timmerman, V., Van Broeckhoven, C., and Jonghe, P. (2001). "Hereditary neuralgic amyotrophy." Neurogenetics. 3: 115-118.

Ørstavik, Kristin, Heier, Mona Skard, Young, Peter, and Stögbauer, Florian. (2001). "Brachial Plexus Involvement as the Only Expression of Hereditary Neuropathy with Liability to Pressure Palsies." Muscle and Nerve, 24: 1093-1096.

Pareyson, Davide. (2003). "Diagnosis of hereditary neuropathies in adult patients." J. Neurol, 250: 148-160.

Pellegrino, J.E., Rebbeck, T.R., Brown, M.J., Bird, T.D., Chance, P.F. (1996). "Mapping of hereditary neuralgic amyotrophy (familial brachial plexus neuropathy) to distal chromosome 17q." Neurology. 46(4): 1128-32.

Pellegrino, Joan E., George, Roberta A.V., Biegel, Jacquelyn, Farlow, Martin R., Gardner, Kathy, Caress, Judy, et al. (1997). "Hereditary neuralgic

amyotrophy: evidence for genetic homogeneity and mapping to chromosome 17q25." Human Genetics, 101: 277-283.

Pennisi, Elizabeth. (2001). "The Human Genome." Science 291(5507): 1177-1180.

QIAGEN, Inc. (2002). QIAquick® Spin Handbook.

Quan, Dianna. (1994). "Neuralgic amyotrophy: Presentation of a case and a review of the syndrome." P&S Medical Review, 1(2), 1-10. 7 Jan. 2004 <http://cpmcnet.columbia.edu/news/review/archives/medrev_v1n2_0002.html>.

Rowland, Lewis P. (Ed.). (1995). Merritt's Textbook Neurology (9th ed.). Baltimore: Williams and Wilkins, a Waverly company.

Sambrook, Fritsch, and Maniatis. (1989). Molecular Cloning: A Laboratory Manual. (2nd ed.) New York City: Cold Spring Harbor Laboratory Press.

Stanford University. (2000). GeneReport for: NM_006640. GeneReport. Retrieved March 14, 2005: http://genome-www5.stanford.edu/cgi-bin/source/sourceResult?option=Number&criteria=NM_006640&choice=Gene.

Stanford University. (2000). GeneReport for: NM_018996. GeneReport. Retrieved March 4, 2005: http://genome-www5.stanford.edu/cgi-bin/source/sourceResult?option=Number&criteria=NM_018996&choice=Gene.

Stanford University. (2000). GeneReport for: NM_022066. GeneReport. Retrieved February 28: http://genome-www5.stanford.edu/cgi-bin/source/sourceResult?option=Number&criteria=Nm_022066&choice=Gene.

Stanford University. (2000). GeneReport for: NM_003258. GeneReport. Retrieved March 14, 2005: http://genome-www5.stanford.edu/cgi-bin/source/sourceResult?option=Number&criteria=Nm_003258&choice=Gene.

Stanford University. (2000). GeneReport for: NM_08414. GeneReport. Retrieved March 14, 2005: http://genome-www5.stanford.edu/cgi-bin/source/sourceResult?option=Number&criteria=Nm_018414&choice=Gene. http://genome-www5.stanford.edu/cgi-bin/source/sourceResult?option=Number&criteria=Nm_003003&choice=Gene.

Stanford University. (2000). GeneReport for: NM_003003. GeneReport. Retrieved March 14, 2005:

Stedman, Thomas. *Stedman's Medical Dictionary* (26th ed.). (1996). Baltimore: Lippincott Williams and Wilkins, A Waverly Co.

Stögbauer, F., Young, P., Timmerman, V., Spoelders, P., Ringelstein, E.B., Van Broeckhoven, C., et al. (1997). "Refinement of the hereditary neuralgic

- amyotrophy (HNA) locus to chromosome 17q24-25." Human Genetics, 99: 685-87.
- Stögbauer, F., Young, P., Kuhlenbaumer, G., Jonghe, P. De, and Timmerman, V. (2000). "Hereditary recurrent focal neuropathies: Clinical and molecular features." Neurology, 54: 546-551.
- Taylor, Richard A. (1960). "Heredofamilial Mononeuritis Multiplex with Brachial Predilection." Brain, 83: 113-137.
- Tsairis, Peter, Dyck, Peter J., and Mulder, Donald W. (1972). "Natural History of Brachial Plexus Neuropathy." Arch Neurology, 27: 109-117.
- Van Alfen, N., and van Engelen, B.G.M. (1997). "Lumbosacral plexus neuropathy: a case report and review of the literature." Clinical Neurology and Neurosurgery, 99: 138-141.
- Van Alfen, N., van Engelen, B. G. M., Reinders, J. W. C., Kremer, H., and Gabreëls, F.J. M. (2000). "The Natural History of Hereditary Neuralgic Amyotrophy in the Dutch Population: Two Distinct Types?" Brain: A Journal of Neurology, 123(4): 718-723.
- Venter J.C., Adams M.D., Myers E.W., Li P.W., Mural R.J., Sutton G.G. et al. (2001). The sequence of the human genome. Science, 291: 1304-1351.
- Watts, G.D.J., O'Brient, K.C., Borreson, T.E., Windebank, A.J., and Chance, P.F. (2001). "Evidence for genetic heterogeneity in hereditary neuralgic amyotrophy." Neurology, 56: 675-678.

Webnox Corp. (2000). Serine Proteases. Hyperdictionary. Retrieved March 2, 2005: <http://www.hyperdictionary.com/search.aspx?define=serine+proteases>.

Windebank, A.J. (1993). Inherited recurrent focal neuropathies. In P. J. Dyke, P.K. Thomas, J.W. Griffin, P.A. Low, J.F. Poduslo (Eds.), Peripheral Neuropathy (Vol. 2, 3rd ed., pp. 1137-1148). Philadelphia, PA: W.B. Saunders Company.