HMG Advance Access published January 12, 2009

Duplication within the *SEPT9* gene associated with a founder effect in North American families with Hereditary Neuralgic Amyotrophy

Megan L. Landsverk[1†], Elizabeth K. Ruzzo[1†], Heather C. Mefford[2], Karen Buysse[2,15], Jillian G. Buchan[1], Evan E. Eichler[2,6] , Elizabeth M. Petty[8], Esther A. Peterson[8], Dana M. Knutzen[1], Karen Barnett[1], Martin R. Farlow[9] , Judy Caress[9], Gareth J. Parry[10], Dianna Quan[11], Kathy L. Gardner[12], Ming Hong[13], Zachary Simmons[14], Thomas D. Bird[3,4,7] , Phillip F. Chance[1,3,5], Mark C. Hannibal[1,5*].

Departments of [1]Pediatrics, [2]Genome Sciences, [3]Neurology, and [4]Medicine, University of Washington School of Medicine; Seattle, WA 98195, USA. [5]Seattle Children's Hospital; Seattle, WA 98105, USA. [6]Howard Hughes Medical Institute; Seattle, WA 98195, USA. [7]Geriatric Research Education and Clinical Center, VA Puget Sound Health Care System; Seattle, WA 98108, USA. [8]Department of Internal Medicine; University of Michigan School of Medicine, Ann Arbor, MI 48105, USA. [9]Department of Neurology; Indiana University School of Medicine, Indianapolis, IN 46202, USA. [10]Department of Neurology; University of Minnesota, Minneapolis, MN 55455, USA. [11]Department of Neurology; University of Colorado Denver, Aurora, CO 80045, USA. [12]Department of Neurology; Veterans Administration Hospital and University of Pittsburgh Medical Center, Pittsburgh, PA 15213, USA. [13]Group Health Bellevue Medical Center; Bellevue, WA 98004, USA. [14]Department of Neurology; Penn State College of Medicine, Hershey Medical Center, Hershey, PA 17033, USA. [15]Center for Medical Genetics, Ghent University Hospital, B-9000, Ghent, Belgium.

[*]Correspondence: mhanni@u.washington.edu; P: 206-221-5465 F: 206-221-5132. HSB RR247 Box 356320, 1959 NE Pacific St, Seattle, WA 98195.

[†]The authors wish it to be known that, in their opinion, the first two authors should be regarded as joint First Authors.

Abstract

Hereditary neuralgic amyotrophy (HNA) is an autosomal dominant disorder associated with recurrent episodes of focal neuropathy primarily affecting the brachial plexus. Point mutations in the *SEPT9* gene have been previously identified as the molecular basis of HNA in some pedigrees. However in many families, including those from North America demonstrating a genetic founder haplotype, no sequence mutations have been detected. We report an intragenic 38 Kb *SEPT9* duplication that is linked to HNA in twelve North American families that share the common founder haplotype. Analysis of the breakpoints showed that the duplication is identical in all pedigrees, and molecular analysis revealed that the duplication includes the 645 bp exon in which previous HNA mutations were found. The *SEPT9* transcript variants that span this duplication contain two in-frame repeats of this exon, and immunoblotting demonstrates larger molecular weight SEPT9 protein isoforms. This exon also encodes for a majority of the *SEPT9* N-terminal proline rich region suggesting that this region plays a role in the pathogenesis of HNA.

Introduction

Hereditary neuralgic amyotrophy (HNA; also called familial brachial plexus neuropathy) is a rare autosomal dominant disorder characterized by attacks of neuropathic pain followed by weakness and atrophy of muscles in the upper extremities (1, 2). Attacks begin with severe, relentless, neuropathic pain that often increases with movement or pressure on the affected limb. The intense pain lasts for about a month on average, and is often followed by persistent musculoskeletal-type pain for weeks to months (3). Muscle weakness generally occurs within the first 24 hours to two weeks after the initial pain attack, and primarily affects the infraspinatus and serratus anterior muscle. Recovery can take months to years, and recent reports indicate that fewer than 25% of patients make a full recovery after three years (3). Almost a quarter of patients suffer their first attack as children. A number of characteristic features have frequently been reported in some, but not all persons with HNA including hypotelorism, shortened palpebral fissures, cleft palate, a long nasal bridge, small oral openings, and epicanthal folds (4, 5). Unusual skin folds and creases have also been observed, usually presenting as ring-shaped skin creases on the necks and limbs of infants (4, 6).

The pathophysiological mechanism of HNA is yet unknown. However, at least 50% of attacks are preceded by events that may alter immune system homeostasis such as stress (emotional and physical), surgery, exposure to cold, infections, immunizations, or pregnancy (3, 7, 8). A number of reports have documented multiple microvessels with epineural perivascular inflammatory infiltrates in nerve biopsies of HNA patients also suggesting an immune response (9, 10).

We previously reported three mutations in the septin 9 gene (*SEPT9)* identified in six HNA families of various ethnic origins (11). At that time, we were unable to detect disease-associated mutations in five additional North American families with HNA (K4000, K4004, K4006, K4007, K4015) by sequencing exons and exon-intron boundaries. However, these individuals all demonstrated linkage to the region containing *SEPT9* on chromosome 17q25, and shared a common disease-linked haplotype providing evidence of a founder effect (12).

SEPT9 is a member of a conserved family of filament-forming GTPases. To date, at least 14 different septin genes have been identified in humans and function in various processes such as cytokinesis, vesicle trafficking, exocytosis, cell polarity and cell motility (13). Through alternative N-terminal splicing, *SEPT9* generates at least seven mRNA transcripts encoding six distinct polypeptides. Mutations linked to HNA have been identified in five transcripts, *SEPT9*_v1, v2, v3, v5 and v6 as named in the NCBI database (NM_001113491, NM_001113493, NM_006640, NM_001113492, and NM_001113494) (Figure 1A) (14). *SEPT9*_v1, v2, and v3 utilize alternative 5' exons generating the three longest polypeptides containing a common proline-rich region and unique N-termini of 25, 18, and 7 amino acids respectively (Figure 1B). These protein isoforms are referred to in the NCBI database as SEPT9a, b and c (NP_001106963, NP_001106965, and NP_006631) (14). However, this nomenclature is confusing as it does not conform to letter designations used in previous SEPT9 publications (15-17). Therefore, we will refer to these protein isoforms according to suggested HGNC guidelines as SEPT9_i1, i2, and i3 to avoid confusion. *SEPT9*_v5 and v6 (also known as v4* and v4) both encode the same polypeptide, SEPT9_i5/6 (listed as SEPT9 isoform e, NP_001106966, in the NCBI database) yet have different 5' UTRs (18). One of the previously identified HNA-linked mutations in *SEPT9* resides in the 5'UTR of *SEPT9*_v3, the other two mutations are located in an exon shared by *SEPT9*_ v1, v2, v3, v5 and v6 (11).

Here we report the identification of seven additional pedigrees (K4002, K4014, K4019, K4021, K4037, K4041, K4042) that contain the disease-associated North American shared haplotype (12). We have identified the mutation responsible for HNA in all twelve shared haplotype families to be a 38 Kb duplication within the *SEPT9* gene. We have found that lymphoblastoid cell lines (LCLs) from affected members of our shared founder haplotype pedigrees express novel SEPT9 immunoreactive protein isoforms that are not present in control or missense mutation containing patients. At least one of these protein products is a result of an in-frame tandem duplication of a 645 bp exon within the duplicated region. Two of the previously identified HNA mutations reside within this exon, suggesting that this region of SEPT9 is involved in the pathogenesis of HNA.

Results

Identification of new HNA pedigrees containing the North American founder mutations

We previously reported five North American HNA affected families that map to chromosome 17q25, and share a common founder haplotype (12). Through linkage and haplotype analysis we have determined that families K4002, K4014, K4019, K4021, K4037, K4041, and K4042 also contain the common shared alleles, including the T allele of SNP rs34587622, present in the founder haplotype (Table 1). In agreement with previously reported shared haplotype families (11), no disease-associated sequence changes in the *SEPT9* gene were identified in affected individuals.

HNA patients possessing the shared haplotype have altered SEPT9 protein products

Recent observations suggest that some SEPT9 isoforms are translationally regulated (19). To determine if SEPT9 translation was altered in HNA families containing the shared haplotype, we analyzed SEPT9 protein expression in LCL lysates from HNA patients, and unaffected family members using an antibody capable of recognizing SEPT9_i1-3 and i5/6 (immunogen noted in Figure 1B). In control patients, the anti-SEPT9 antibody detected an immunoreactive species that migrated at around 75 kDa, with shorter exposures indicating a doublet (data not shown). These results are consistent with previous reports for the molecular weights of SEPT9_i1, i2, and i3 (20, 21). This same doublet was observed in patients possessing the previously identified NM_006640.4:c.262C>T (R88W) and NM_006640.4:c.278C>T (S93F) HNA mutations, suggesting that these point mutations do not alter the molecular weights of SEPT9_i1, i2, or i3. We found that lysates from HNA patients sharing the common founder haplotype expressed SEPT9 reactive protein products absent in control or known point-mutation containing patients. Two additional species migrating at ~100 and ~80 kDa were consistently observed, and a third species migrating slightly faster than the ~100 kDa band was often seen in some patients (Figure 2). Using an antibody specific to SEPT9_i1, we determined that not only the band migrating at ~75 kDa, but also the band migrating at ~100 kDa were SEPT9_i1 specific (middle row Figure 2).

To determine if SEPT9 isoforms were altered at the mRNA level, we isolated RNA from control and affected patient samples and performed RT-PCR using *SEPT9*_v1, v2, and v3 specific forward primers and a common reverse primer (Figure 3A). No gross alterations in message were observed (Figure 3B). However, in addition to the expected PCR product, HNA affected patients with the shared haplotype often showed faint bands approximately 650 bp larger than the wild-type band (Figure 3C). Because this increase in size was similar to the size of the exon where previously identified HNA mutations have been found, we analyzed expression of that particular exon only. As can be seen in Figure 3D, an extra 950 bp band was observed in cDNA from HNA shared haplotype patients but not in controls using primers found only in that exon, suggesting the presence of two copies of this exon in tandem orientation. To confirm the presence of two tandem copies of the 645 bp exon, we utilized a reverse primer spanning the putative breakpoint with the same forward primers as in Figure 3A. As can be observed in Figure 3E, only patients containing the founder haplotype express *SEPT9*_v1, v2, and v3 with two tandem copies of the 645 bp exon. Figure 3F shows an example of the putative protein product generated by the addition of this exon into SEPT9_i1.

Pedigrees containing the shared haplotype contain a 38 Kb tandem duplication within the *SEPT9* gene

To search for a possible genomic alteration in HNA shared haplotype subjects that might explain the altered cDNA and protein products, we performed array comparative genomic hybridization (CGH) on individuals from pedigrees K4000 and K4015 using a high-density oligonucleotide array targeted to the *SEPT9* locus. Our analysis identified a 38 Kb duplication within *SEPT9* (hg18/Build 35 chr17:72,876,638-72,914,241) (Figure 4). This duplication encompassed the 645 bp exon containing previously identified HNA mutations, as well as the first exons of *SEPT9*_v2 and v6. Copy number variations within the *SEPT9* gene have not been previously described (see http://projects.tcag.ca/variation). In addition, we have previously analyzed 39 healthy controls using the same high-density array design, and none have copy number changes within the *SEPT9* gene (data not shown).

To verify the breakpoint between the duplicated regions, we designed forward (red arrows Figure 5A) and reverse (blue arrows Figure 5A) PCR primers located in unique regions on either side of the putative break point. This strategy generated an approximately 1.5 Kb PCR product in HNA patients with the shared founder haplotype, but not in control (n=102) or *SEPT9* point-mutation containing patients (n=10) (Figure 5B). To control for presence and quality of the DNA, *SEPT9*_v1 exon 2 was amplified using the same PCR conditions. The 1.5 Kb PCR product was sequenced, and confirmed the presence of a tandem duplication (Figure 5C). Further analysis of all individuals in the founder haplotype pedigrees shows that the duplication does indeed segregate with the disease (Figure 6; Supplementary Figure 1A and B). The penetrance of neurological attacks in these families is approximately 95%, which is consistent with previous reports (22). However, for many pedigrees recent clinical data was not available, and therefore, those individuals were not considered in penetrance calculations.

The proximal breakpoint of the duplication is located at the 3' end of an AluSx repetitive element. Despite the presence of several Alu elements in the region, the distal breakpoint is in unique sequence that does not have sequence homology to the AluSx repeat at the proximal breakpoint. Because of the presence of multiple Alu elements near both breakpoints, we performed RepeatMasker analysis (www.repeatmasker.org) of the *SEPT9* gene locus which revealed 124 Alu elements within the gene (15% of sequence, 1 Alu/1.8 kb), slightly higher than the genome average of ~10% (23).

Confirmation of an in-frame duplication of the 645 bp exon in all shared haplotype families

To confirm that individuals with altered protein products all shared the 645 bp exon tandem duplication, we performed RT-PCR using a forward primer in the 3' (red arrows Figure 7A) region of this exon and a reverse primer in the 5’ region (blue arrows Figure 7A). As can be seen in Figure 7B, all HNA affected individuals with a larger SEPT9 isoform a protein product were positive for a tandem duplication of this exon at the mRNA level. The presence of two tandem copies of the 645 bp exon was confirmed through sequence analysis (Figure 7C).

Discussion

We identified a duplication within the *SEPT9* gene that is linked to HNA in families that share a common founder haplotype, providing further evidence for a single founder effect alteration. The families with the duplication account for almost 25% of our cohort of HNA pedigrees that link to chromosome 17q25. The 38 Kb duplication includes the exon in which two of the three previously identified HNA-linked mutations, NM_006640.4:c.262C>T and NM_006640.4:c.278C>T, are located. Affected patients from these founder pedigrees express *SEPT9* transcripts containing in-frame tandem repeats of this exon and produce larger protein products. At least one of these proteins is a larger version of SEPT9 isoform 1.

In SEPT9_i1, i2, and i3 isoforms, the 645 bp exon encodes a majority of the N-terminal proline-rich region. The function of this region is unknown. However, it appears to play a role in SEPT9 filament formation since overexpression constructs of SEPT9_i1 and i3 assemble into filaments (16, 21), while the shorter SEPT9_i5/6, which does not contain the N-terminal extension, does not form filaments (21, 24). The presence of the two HNA point missense mutations does not affect the ability of SEPT9_i3 to assemble into filaments, yet appears to block filament disassembly by the Rho signaling pathway (25). Briefly, overexpression of SA-RhoGEF, constitutively active Rho, or the Rho effector Rhotekin disrupt SEPT9_i3 filaments (17, 26). However, assembled SEPT9_i3 filaments containing the two HNA missense mutations are resistant to disruption by Rho and Rhotekin (25) suggesting that HNA mutations may stabilize SEPT9 filaments.

How alterations in *SEPT9* lead to HNA remains unknown. There are no apparent clinical differences between the families with point mutations in the 645 bp exon and those having the duplication. Interestingly, the family containing a mutation in the 5’ UTR of *SEPT9*_v3 does not have the dysmorphic features observed in other HNA pedigrees (11), suggesting that the region encompassed by the 645 bp exon is important for craniofacial development. Because antibodies that are specific to SEPT9_i2, i3, and i5/6 are not currently available, we cannot identify the other SEPT9 protein products present in the shared haplotype families. Based on molecular weight, the novel 80 kD band is predicted to be SEPT9_i5/6 possessing two copies of the 645 bp exon. However, the wild-type isoform of SEPT9_i5/6 migrates at approximately 60 kD (20) and was not observed in LCLs from either shared haplotype or unaffected patients. It is possible that SEPT9_i5/6 wild-type expression levels are too low to detect, and that the mutant protein is stabilized by the addition of amino acids encoded by the duplicated exon. Since *SEPT9* variants are expressed in a variety of tissue types (27), it is possible that the isoform(s) responsible for HNA are not the same as those expressed in patient LCLs. Recent work by McDade et al. shows that *SEPT9*_v6 (also known as v4) transcripts containing the 262C>T mutation in the 5’ UTR are translated much more efficiently than wild-type under hypoxic conditions, leading to overexpression of SEPT9_i5/6 (19).

Therefore, HNA mutations may affect the function of particular *SEPT9* variants only under conditions of cellular stress.

The proximal breakpoint of the founder duplication mutation was located adjacent to an Alu element, and we noted several Alu elements near the distal breakpoint. The density of Alu elements in the *SEPT9* gene (15%) is higher than the genome average, which may result in increased genomic instability and susceptibility to rearrangement. There are several examples of genes with an increased density of Alu repeats in which multiple deletion and/or duplication mutations have been reported, including *LDLR* in familial hypercholesterolemia (28, 29) and *LIS1* in lissencephaly (30). Interestingly, our preliminary data suggests that additional copy number changes in the *SEPT9* gene are found in HNA families in which sequence analysis has failed to identify the causative mutation (Hannibal M, unpublished data).

In summary, our analysis of 12 North American HNA pedigrees harboring an apparent founder chromosome have an in-frame, 38 kb tandem duplication encompassing a 645 bp exon within the *SEPT9* gene in which previous HNA mutations were found. Analysis of the breakpoints showed that the duplication is identical in all pedigrees, may have been mediated by Alu elements and is likely non-recurrent. Together, the three previously reported point mutations and shared haplotype duplication account for almost half of our cohort of HNA families (Hannibal et al., manuscript submitted), and we predict that copy number changes within *SEPT9* will account for a significant fraction of families with previously unexplained HNA. Neuralgic amyotrophy (NA) also exists as a sporadic disorder known as idiopathic neuralgic amyotrophy (INA) or Parsonage-Turner syndrome (31). Compared to HNA, INA attacks have a higher incidence rate, usually occur later in life and recurrences are less frequent (3). However, given the degree of clinical overlap, it is possible that HNA and INA may share a common pathogenesis. Analysis of HNA offers a genetic approach necessary to gain insights into the more common sporadic disorder.

Methods and Materials

Subjects

Twelve pedigrees with HNA (K4000, K4002, K4004, K4006, K4007, K4014, K4015, K4019, K4021, K4037, K4041, and K4042) were diagnosed according to published criteria (1). Linkage to chromosome 17q25, and clinical features of affected individuals in pedigrees K4000, K4004, K4006, K4007, and K4015 has been previously described (32-34). A number of individuals in pedigrees K4002, K4014, K4019, K4021, K4037, K4041, and K4042 were also noted to possess the physical characteristics previously associated with HNA (4).

Genomic DNA extraction and genotyping

Blood samples were obtained by venipuncture under a protocol of informed consent (Human Subjects Division, University of Washington, Seattle). Genomic DNA was extracted as previously described (12). Short tandem repeat (STR) markers were utilized, as described previously (11, 12) to genotype individuals in new pedigrees (K4002, K4014, K4019, K4021, K4037, K4041, and K4042).

Array CGH

Array comparative genomic hybridization (CGH) was performed using a custom oligonucleotide array consisting of 385,000 isothermal probes (NimbleGen Systems, Madison, WI). The array included 1311 probes spanning a 300-kb region encompassing the *SEPT9* gene (chr17: 72,800,000-73,100,000; average probe spacing 1 probe / 229bp). Hybridizations were carried out as described previously (35) using a single normal male as reference (GM15724, Coriell, Camden, NJ).

DNA sequencing and RT-PCR

Oligonucleotide primers designed to unique regions on either side of the predicted breakpoint region were used to PCR amplify a junction fragment. Permanent lymphoblastoid cell lines (LCLs) were established through Epstein-Barr virus transformation and maintained under standard conditions (36). Total RNA was extracted from LCLs using an RNeasy mini kit (Qiagen). RT-PCR was performed using Superscript III polymerase (Invitrogen) and an oligo-dT primer, according to manufacturer’s instructions. PCR products were purified using a QIAquick kit (Qiagen), and sequenced by the DNA Sequencing Facility at the University of Washington, Department of Biochemistry. Primer sequences are available upon request.

Western blots

LCLs from HNA affected patients and unaffected family members were lysed in 150 mM NaCl, 50 mM Tris-HCl pH 7.6, 1% Triton X-100 plus protease inhibitors. Samples were run on 7.5% SDS-PAGE gels, transferred to PVDF membranes, and probed with anti-SEPT9 isoforms 1-3 and 5/6 reactive antibodies or an anti-SEPT9_i1 specific antibody as previously described (37). An anti-actin antibody (Sigma) was used as a control.

Funding

The work was supported by funds from the National Institutes of Heath (NINDS), [grant number NS38181 to PFC and MCH]; The Neuropathy Association, New

York, New York, [to PFC]; and the Allan and Phyllis Treuer Endowed Chair for Genetics and Development [to PFC].

Acknowledgements

We thank Jonathan Adkins and Melissa Eckert for DNA isolation and lymphoblastoid cell line establishment. We thank all of the clinical contributors, patients and families for facilitating pedigree identification and obtaining research materials. Specifically, we thank Drs. Eva Feldman, Melanie Bedford and Brian Tsao for providing samples. KB is a research assistant of the Research Foundation – Flanders (FWO-Vlaaderen).

Conflicts of Interest

The authors declare that they have no conflicting interests.

References

1 Kuhlenbäumer, G., Stögbauer, F., Timmerman, V. and De Jonghe, P. (2000) Diagnostic guidelines for hereditary neuralgic amyotrophy or heredofamilial neuritis with brachial plexus predilection. On behalf of the European CMT Consortium. *Neuromuscul. Disord.*, **10**, 515-517.

2 Hannibal, M.C., van Alfen, N., Chance, P.F. and van Engelen, B.G. (2008), In *GeneReviews at GeneTests: Medical Genetics Information Resource*. Copyright, University of Washington, Seattle. 1997-2008. Available at http://www.genetests.org.

3 van Alfen, N. and van Engelen, B.G. (2006) The clinical spectrum of neuralgic amyotrophy in 246 cases. *Brain*, **129**, 438-450.

4 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**, 1963-1968.

5 Gardner, J.H. and Maloney, W. (1968) Hereditary brachial and cranial neuritis genetically linked with ocular hypotelorism and syndactyly. *Neurology*, **18**, 278.

6 Bass, H.N., Caldwell, S. and Brooks, B.S. (1993) Michelin tire baby syndrome: familial constriction bands during infancy and early childhood in four generations. *Am. J. Med. Genet.*, **45**, 370-372.

7 Tsairis, P., Dyck, P.J. and Mulder, D.W. (1972) Natural history of brachial plexus neuropathy. Report on 99 patients. *Arch. Neurol.*, **27**, 109-117.

8 Bardos, V. and Somodska, V. (1961) Epidemiologic study of a brachial plexus neuritis outbreak in northeast Czechoslovakia. *World. Neurol.*, **2**, 973-979.

9 Klein, C.J., Dyck, P.J., Friedenberg, S.M., Burns, T.M., Windebank, A.J. and Dyck, P.J. (2002) Inflammation and neuropathic attacks in hereditary brachial plexus neuropathy. *J. Neurol. Neurosurg. Psychiatry*, **73**, 45-50.

10 Suarez, G.A., Giannini, C., Bosch, E.P., Barohn, R.J., Wodak, J., Ebeling, P., Anderson, R., McKeever, P.E., Bromberg, M.B. and Dyck, P.J. (1996) Immune brachial plexus neuropathy: suggestive evidence for an inflammatory-immune pathogenesis. *Neurology*, **46**, 559-561.

11 Kuhlenbäumer, G., Hannibal, M.C., Nelis, E., Schirmacher, A., Verpoorten, N., Meuleman, J., Watts, G.D., De Vriendt, E., Young, P., Stögbauer, F. *et al.* (2005) Mutations in *SEPT9* cause hereditary neuralgic amyotrophy. *Nat. Genet.*, **37**, 1044-1046.

12 Watts, G.D., O'Briant, K.C. and Chance, P.F. (2002) Evidence of a founder effect and refinement of the hereditary neuralgic amyotrophy (HNA) locus on 17q25 in American families. *Hum. Genet.*, **110**, 166-172.

13 Hall, P.A. and Russell, S.E. (2004) The pathobiology of the septin gene family. *J. Pathol.*, **204**, 489-505.

14 Pruitt, K.D., Tatusova, T., Klimke, W. and Maglott, D.R. (2008) NCBI Reference Sequences: current status, policy and new initiatives. *Nucleic Acids Res*.

15 Nagata, K., Asano, T., Nozawa, Y. and Inagaki, M. (2004) Biochemical and cell biological analyses of a mammalian septin complex, Sept7/9b/11. *J. Biol. Chem.*, **279**, 55895-55904.

16 Nagata, K., Kawajiri, A., Matsui, S., Takagishi, M., Shiromizu, T., Saitoh, N., Izawa, I., Kiyono, T., Itoh, T.J., Hotani, H. *et al.* (2003) Filament formation of MSF-A, a mammalian septin, in human mammary epithelial cells depends on interactions with microtubules. *J. Biol. Chem.*, **278**, 18538-18543.

17 Nagata, K. and Inagaki, M. (2005) Cytoskeletal modification of Rho guanine nucleotide exchange factor activity: identification of a Rho guanine nucleotide exchange factor as a binding partner for Sept9b, a mammalian septin. *Oncogene*, **24**, 65-76.

18 McIlhatton, M.A., Burrows, J.F., Donaghy, P.G., Chanduloy, S., Johnston, P.G. and Russell, S.E. (2001) Genomic organization, complex splicing pattern and expression of a human septin gene on chromosome 17q25.3. *Oncogene*, **20**, 5930-5939.

19 McDade, S.S., Hall, P.A. and Russell, S.E. (2007) Translational control of SEPT9 isoforms is perturbed in disease. *Hum. Mol. Genet.*, **16**, 742-752.

20 Surka, M.C., Tsang, C.W. and Trimble, W.S. (2002) The mammalian septin MSF localizes with microtubules and is required for completion of cytokinesis. *Mol. Biol. Cell*, **13**, 3532-3545.

21 Robertson, C., Church, S.W., Nagar, H.A., Price, J., Hall, P.A. and Russell, S.E. (2004) Properties of SEPT9 isoforms and the requirement for GTP binding. *J. Pathol.*, **203**, 519-527.

22 Meulemann, J., Kuhlenbäumer, G., Schirmacher, A., Wehnert, M., De Jonghe, P., De Vriendt, E., Young, P., Airaksinen, E., Pou-Serradell, A., Prats, J.M. *et al.* (1999) Genetic refinement of the hereditary neuralgic amyotrophy (HNA) locus at chromosome 17q25. *Eur. J. Hum. Genet.*, **7**, 920-927.

23 Lander, E.S., Linton, L.M., Birren, B., Nusbaum, C., Zody, M.C., Baldwin, J., Devon, K., Dewar, K., Doyle, M., FitzHugh, W. *et al.* (2001) Initial sequencing and analysis of the human genome. *Nature*, **409**, 860-921.

24 Chacko, A.D., Hyland, P.L., McDade, S.S., Hamilton, P.W., Russell, S.H. and Hall, P.A. (2005) *SEPT9*_v4 expression induces morphological change, increased motility and disturbed polarity. *J. Pathol.*, **206**, 458-465.

25 Sudo, K., Ito, H., Iwamoto, I., Morishita, R., Asano, T. and Nagata, K. (2007) *SEPT9* sequence alternations causing hereditary neuralgic amyotrophy are associated with altered interactions with SEPT4/SEPT11 and resistance to Rho/Rhotekin-signaling. *Hum. Mutat.*, **28**, 1005-1013.

26 Ito, H., Iwamoto, I., Morishita, R., Nozawa, Y., Narumiya, S., Asano, T. and Nagata, K. (2005) Possible role of Rho/Rhotekin signaling in mammalian septin organization. *Oncogene*, **24**, 7064-7072.

27 Scott, M., Hyland, P.L., McGregor, G., Hillan, K.J., Russell, S.E. and Hall, P.A. (2005) Multimodality expression profiling shows *SEPT9* to be overexpressed in a wide range of human tumours. *Oncogene*, **24**, 4688-4700.

28 Nissen, P.H., Damgaard, D., Stenderup, A., Nielsen, G.G., Larsen, M.L. and Faergeman, O. (2006) Genomic characterization of five deletions in the LDL

receptor gene in Danish Familial Hypercholesterolemic subjects. *BMC Med. Genet.*, **7**, 55.

29 Lehrman, M.A., Goldstein, J.L., Russell, D.W. and Brown, M.S. (1987) Duplication of seven exons in LDL receptor gene caused by Alu-Alu recombination in a subject with familial hypercholesterolemia. *Cell*, **48**, 827-835.

30 Mei, D., Lewis, R., Parrini, E., Lazarou, L.P., Marini, C., Pilz, D.T. and Guerrini, R. (2008) High frequency of genomic deletions--and a duplication--in the LIS1 gene in lissencephaly: implications for molecular diagnosis. *J. Med. Genet.*, **45**, 355-361.

31 Parsonage, M.J. and Turner, J.W.A. (1948) Neuralgic amyotrophy. The shoulder girdle syndrome. *Lancet*, **1**, 973-978.

32 Pellegrino, J.E., George, R.A., Biegel, J., Farlow, M.R., Gardner, K., Caress, J., Brown, M.J., Rebbeck, T.R., Bird, T.D. and Chance, P.F. (1997) Hereditary neuralgic amyotrophy: evidence for genetic homogeneity and mapping to chromosome 17q25. *Hum. Genet.*, **101**, 277-283.

33 Watts, G.D., O'Briant, 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.

34 Taylor, R.A. (1960) Heredofamilial mononeuritis multiplex with brachial predilection. *Brain*, **83**, 113-137.

35 Selzer, R.R., Richmond, T.A., Pofahl, N.J., Green, R.D., Eis, P.S., Nair, P., Brothman, A.R. and Stallings, R.L. (2005) Analysis of chromosome breakpoints in neuroblastoma at sub-kilobase resolution using fine-tiling oligonucleotide array CGH. *Genes Chromosomes Cancer*, **44**, 305-319.

36 Neitzel, H. (1986) A routine method for the establishment of permanent growing lymphoblastoid cell lines. *Hum. Genet.*, **73**, 320-326.

37 Gonzalez, M.E., Peterson, E.A., Privette, L.M., Loffreda-Wren, J.L., Kalikin, L.M. and Petty, E.M. (2007) High *SEPT9*_v1 expression in human breast cancer cells is associated with oncogenic phenotypes. *Cancer Res.*, **67**, 8554-8564.

Legends to Figures

Figure 1. HNA-linked mutations are found in multiple *SEPT9* transcripts. (A) The *SEPT9* gene is approximately 220 Kb in size (hash marks denote 50 Kb intervals) and produces at least seven different mRNA transcripts encoding six different polypeptides. [Note, not all exons are to scale.] However, only *SEPT9*_v1, v2, v3, v5, and v6 contain known HNA mutations. Coding regions are indicated in black. HNA mutations are marked with asterisks. Two mutations are in both the coding regions of *SEPT9*_v1, v2, v3 (R88W and S93F) and the 5' UTR regions of *SEPT9*_v5 and v6 (also known as v4* and v4). A third is located in the 5'UTR of *SEPT9*_v3. (B) Domain structure of SEPT9 polypeptides. All SEPT9 isoforms posses a polybasic domain (shown in green) followed by a GTP-binding domain at the C-terminus. SEPT9 isoforms 1-3 also contain an N-terminal proline-rich extension. HNA mutations are indicated with arrowheads. Regions representing immunogens for SEPT9_i1-3, i5/6 and SEPT9_i1 antibodies are shown in black bars and labeled (1) and (2) respectively.

Figure 2. Individuals possessing the shared haplotype express altered SEPT9 protein products. Lymphoblastoid cells from unaffected (K4035 and K4041), HNA affected founder haplotype (K4000, K4004, K4006, K4007, K4014, K4015, K4019, K4021, K4037, K4041, and K4042) and HNA affected point mutation (K4052 and K4018) individuals were lysed and probed with antibodies to SEPT9_i1-3 and i5/6 or SEPT9_i1 only. Individuals possessing the founder haplotype express novel SEPT9 reactive bands at ~80 and ~100 kDa. A

SEPT9_i1 specific antibody shows that one of the bands at 100 kDa and one at 74 kDa are SEPT9 _i1 reactive. Actin expression serves as a loading control.

Figure 3. Altered SEPT9 protein products are a result of an in-frame tandem duplication of a 645 bp exon. (A) Primer sites in *SEPT9* transcripts used for RT-PCR are indicated by arrowheads. (B) Lymphoblastoid cells from unaffected and HNA affected founder haplotype individuals express wild-type copies of *SEPT9*_v1, v2, and v3. (C) A minor band 650 bp larger than the expected wild-type product was often observed in founder haplotype individuals. This increase in size is the same as that of the exon containing previously identified HNA mutations. (D) PCR using primers within the 645 bp exon produces a larger minor band only in founder haplotype cDNA, suggesting two copies of the exon in tandem. (E) PCR using a reverse primer spanning the tandem duplication shows that individuals possessing the founder haplotype express *SEPT9_* v1, v2 and v3 with two copies of the 645 bp exon. Forward primers in (E) are the same as those used in (A). (F) Model of SEPT9_i1 containing two copies of the 645 bp exon.

Figure 4. Identification of a 38 Kb intragenic duplication within *SEPT9.* Array CGH results from two individuals possessing the HNA founder haplotype show a duplicated region within *SEPT9.* Dotted lines indicate proximal and distal breakpoints. The duplication encompasses the first exons of *SEPT9* transcript

variants 2 and 6 as well as the 645 bp exon in which two of the previous HNA mutations have been identified (marked with asterisks).

Figure 5. Characterization of *SEPT9* intragenic duplication. (A) To further refine the *SEPT9* duplicated region, primers were designed to span the duplication junction. Sites of primers are noted in red and blue arrowheads. The presence of the tandem duplication yields a PCR product of ~2 Kb. An exon outside of the duplicated region was used as a control (green arrowheads). The size of the *SEPT9* gene containing the duplication is approximately 290 Kb. (B) Genomic DNA from unaffected (K4035 and K4041), HNA affected founder haplotype (K4000, K4004, K4006, K4007, K4014, K4015, K4019, K4021, K4037, K4041, K4042, and K4002) and HNA affected point mutation (K4052 and K4018) individuals was screened for the presence of the intragenic duplication. No LCLs were available for pedigree K4002. However, this family shares the common founder haplotype, and the same duplication was confirmed through genomic PCR. (C) Sequence analysis of the duplication breakpoint from two individuals with the founder haplotype. The highlighted nucleotide shows the proximal end of the duplication.

Figure 6. Segregation analysis of founder haplotype family K4041. PCR analysis of the genomic breakpoint in all members of pedigree K4041 demonstrates consistent segregation of the disease with the duplication. *Circles* females, *squares* males, *Empty symbols* unaffected individuals, *filled symbols*

affected individuals, *cross-slashed symbols* deceased, *dotted symbols* non-penetrant carriers.

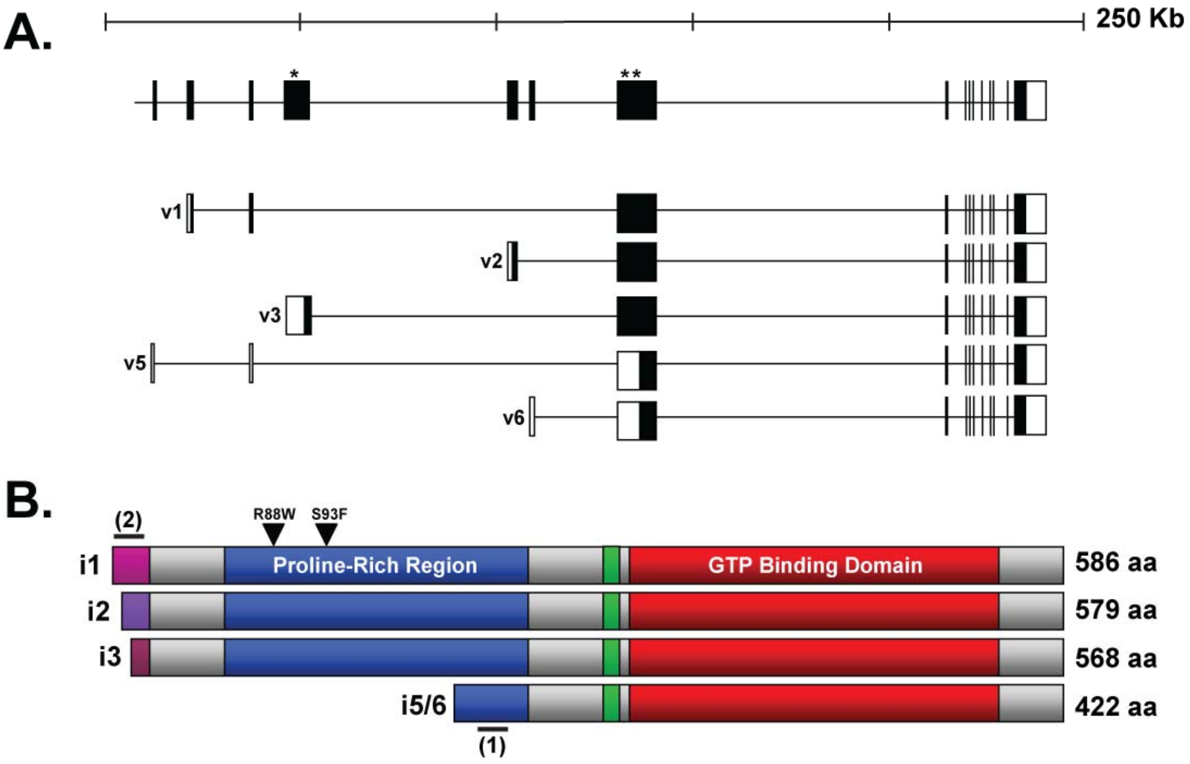
Figure 7. Affected HNA individuals in all shared haplotype pedigrees have an in-frame tandem duplication of the 645 bp exon. (A) To confirm the presence of in-frame tandem copies of the 645 bp exon, a forward primer (red arrowhead) at the 3’ end and a reverse primer (blue arrowhead) at the 5’ end of the exon were designed. The presence of tandem copies of the 645 bp exon yields a PCR product of 597 bp. Exons outside of the duplicated region were used as a control (green arrowheads). The wild-type size of the *SEPT9*_v1, v2, and v3 coding regions is 1685 bp plus alternate 5’ exons. The addition of the 645 bp exon in tandem increases this size to 2330 bp plus alternate 5’ exons. (B) cDNA from unaffected (K4035 and K4041), HNA affected founder haplotype (K4000, K4004, K4006, K4007, K4014, K4015, K4019, K4021, K4037, K4041, and K4042) and HNA affected point mutation (K4052 and K4018) individuals was screened from the presence of tandem copies of the 645 bp exon. (C) Sequence analysis confirms that the tandem duplication of the 645 bp exon remains in-frame. The highlighted nucleotide indicates the 3’ end of the exon.

Table 1. HNA pedigrees sharing the founder haplotype. Genotyping analysis shows that HNA family pedigrees K4002, K4014, K4019, K4021, K4037, K4041, and K4042 share the same founder haplotype as previously published pedigrees (12). Gray boxes represent shared alleles, dashes indicate data not available. The smallest common region is between microsatellite markers 72GT2 and GT6. Markers MSFTri through GT1 lie within the *SEPT9* gene.

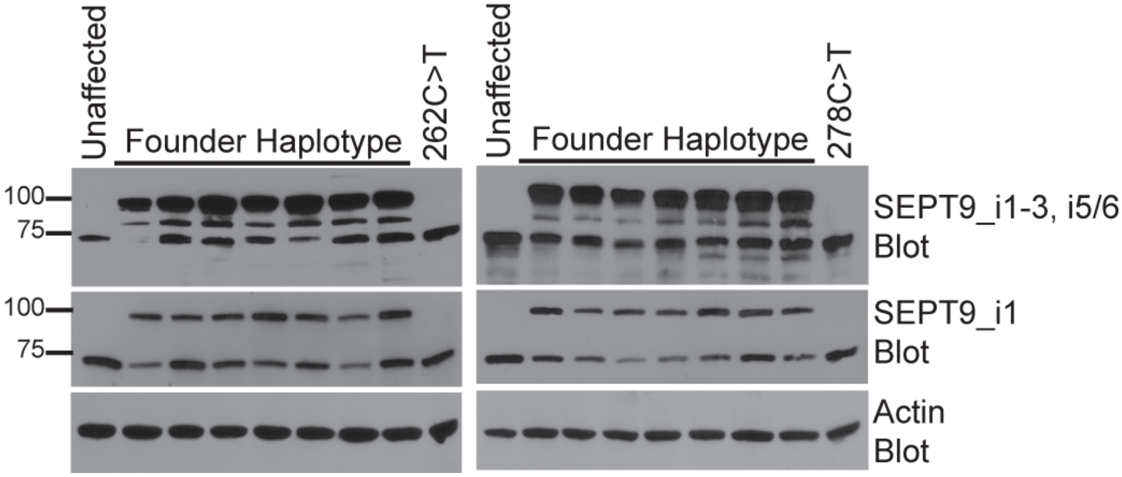
Table 1

Marker or SNP	Previously Reported Pedigrees					New Founder Haplotype Pedigrees						
	K4000	**K4004**	**K4006**	**K4007**	**K4015**	**K4002**	**K4014**	**K4019**	**K4021**	**K4037**	**K4041**	**K4042**
D17S1603	5	1	5	8	5	5	5	-	3	3	1	3
D17S785	3	6	3	3	3	3	3	-	8	4	6	-
D17S801	3	15	3	3	3	-	-	-	-	4	13	14
D17S722	5	7	5	5	5	-	5	5	7	5	4	5
72GT2	5	5	5	5	5	-	-	-	5	-	-	-
72GT1	5	5	5	5	5	-	-	-	5	5	5	5
MSFtri	6	6	6	6	6	6	-	-	6	6	6	6
D17S937	7	7	7	7	7	-	7	7	7	7	7	7
rs34587622	T	T	T	T	T	T	T	T	T	T	T	T
380058	3	3	3	3	3	3	-	-	3	3	3	3
D17S939	1	1	1	1	1	1	1	1	1	1	1	1
GT1	6	6	6	6	6	6	6	6	6	6	6	6
GT6	2	2	2	2	2	2	2	2	2	2	2	2
CA5	4	4	2	4	4	4	4	-	4	4	4	4
D17S802	7	7	5	7	7	7	7	7	7	7	7	7

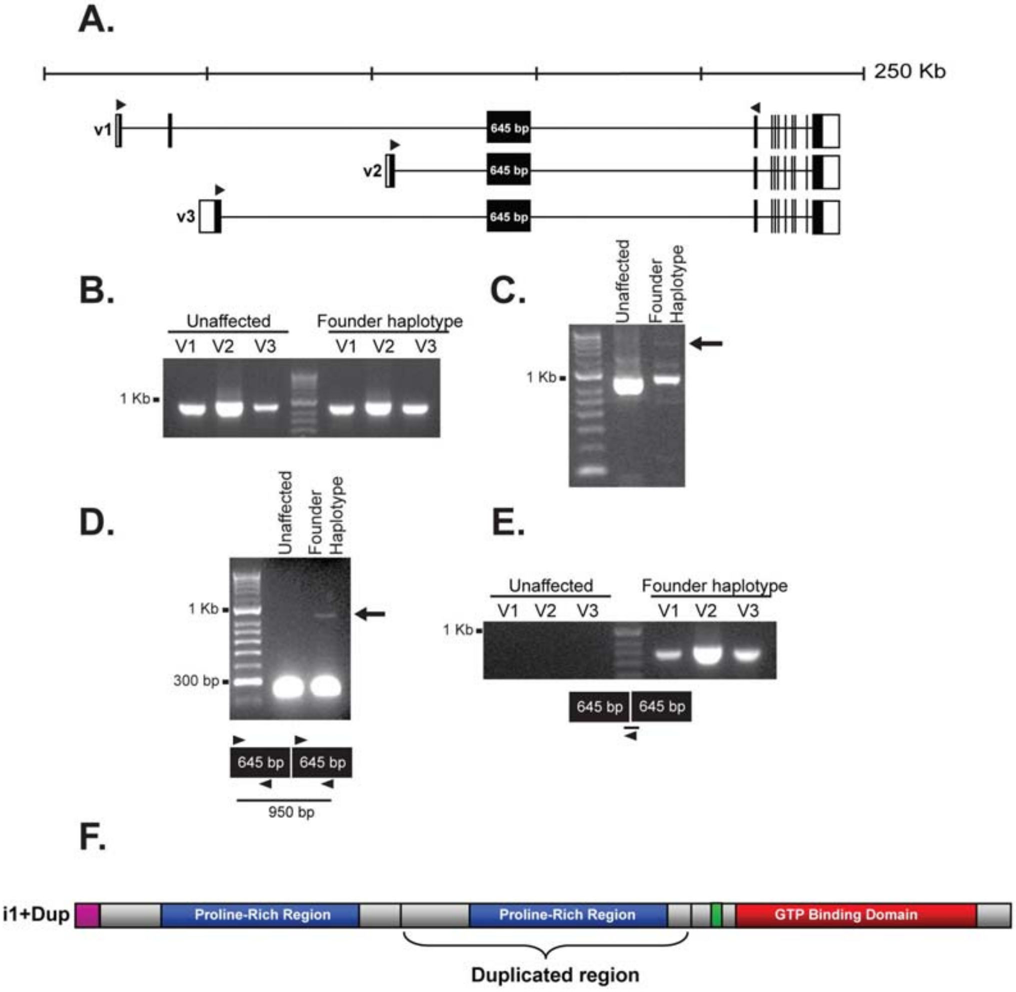
Landsverk et al.
Figure 1



Landsverk et al.
Figure 2

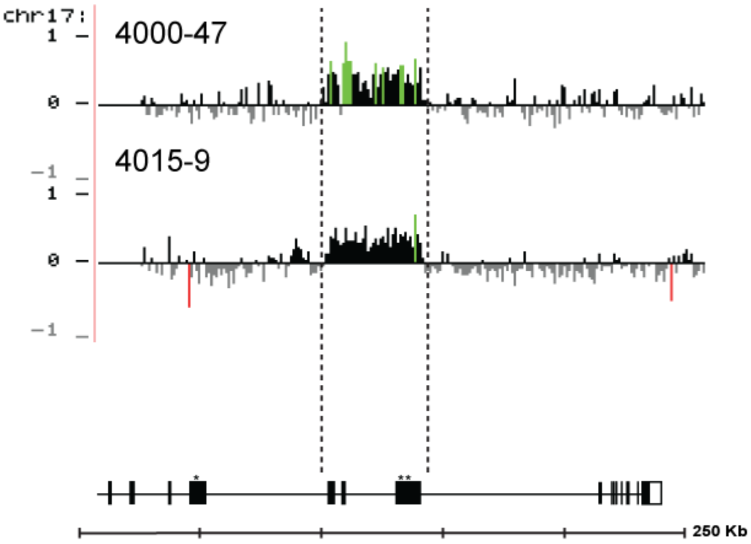


Landsverk et al.
Figure 3

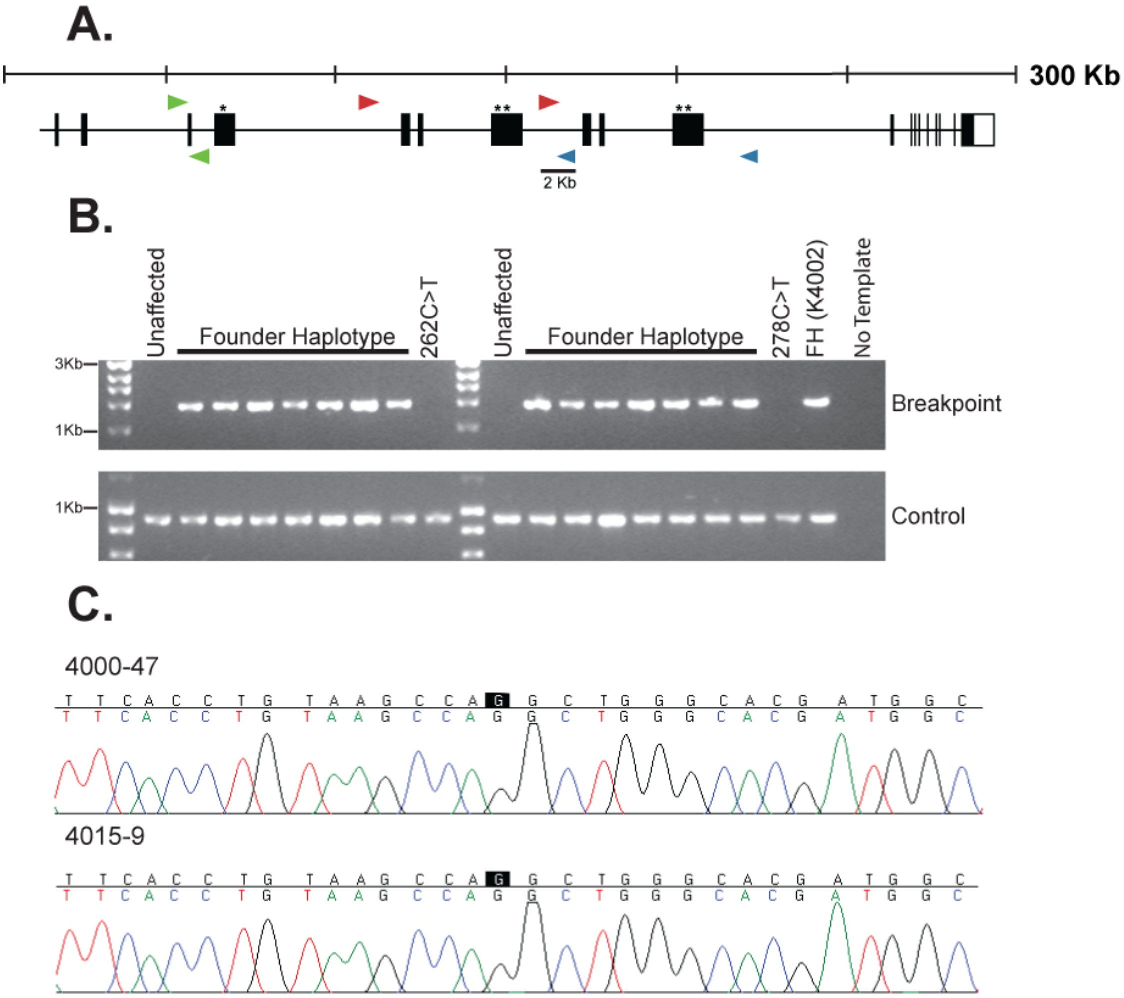


Landsverk et al.

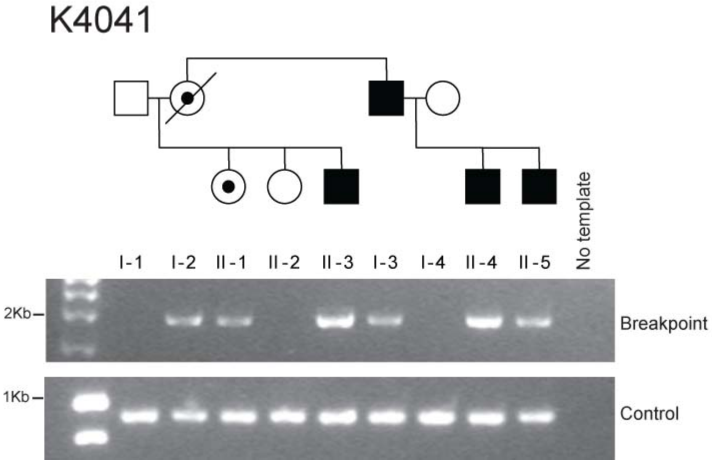
Figure 4



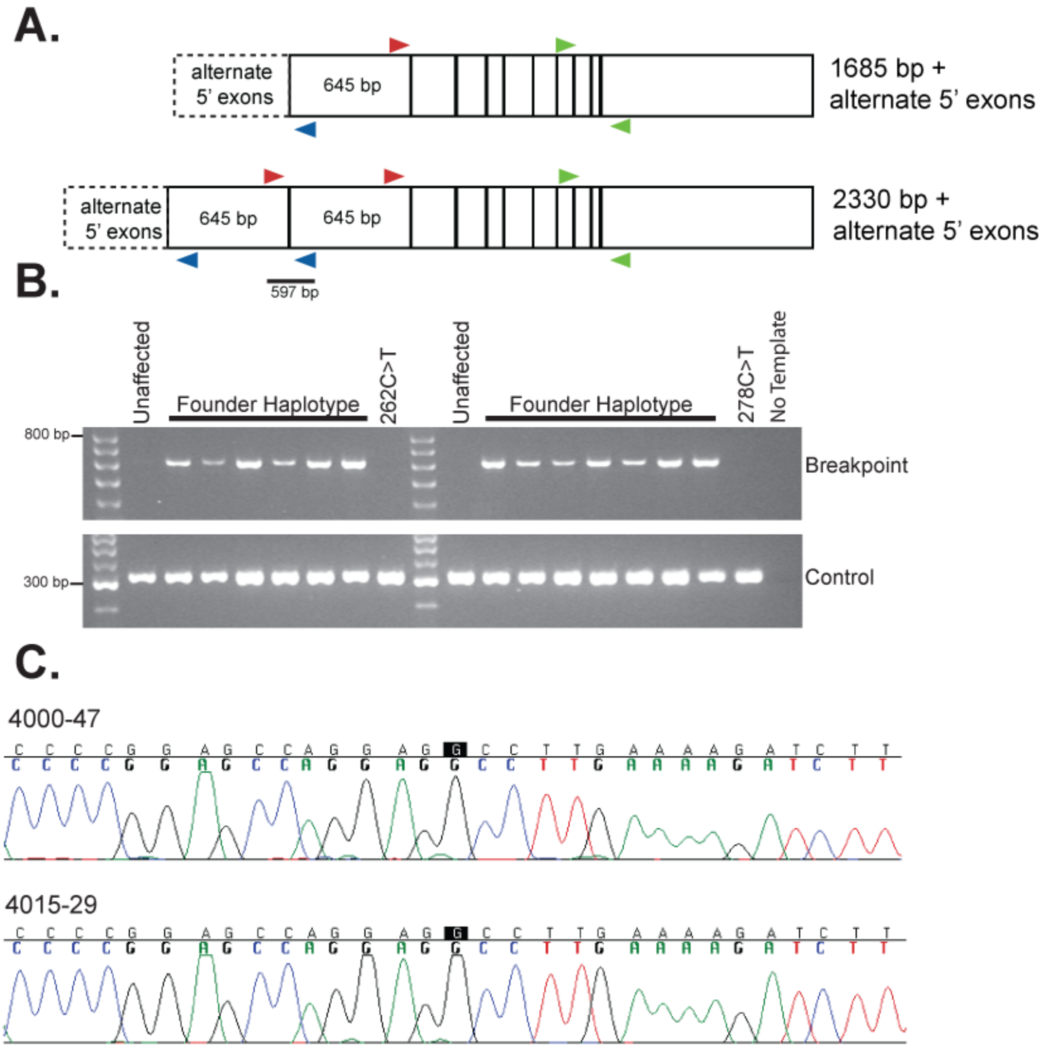
Landsverk et al.
Figure 5



Landsverk et al.
Figure 6



Landsverk et al.
Figure 7



Abbreviations: HNA, Hereditary Neuralgic Amyotrophy; LCL, lymphoblastoid cell line; STR, short tandem repeat; CGH, comparative genomic hybridization; NA, Neuralgic amyotrophy; INA, Idiopathic Neuralgic Amyotrophy.