Duplication within the SEPT9 gene associated with a founder effect in North American families with hereditary neuralgic amyotrophy

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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 12 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

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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 h to 2 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 3 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 and 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) (Fig. 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 (Fig. 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 and K4042) that contain the disease-associated North American shared haplotype (12).
be a 38 Kb duplication within the \textit{SEPT9} gene. We have found that lymphoblastoid cell lines (LCLs) from affected members of our shared founder haplotype pedigrees express novel \textit{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 \textit{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 \textit{SEPT9} gene were identified in affected individuals.

<table>
<thead>
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<th>Marker or SNP</th>
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<th>New founder haplotype pedigrees</th>
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<tr>
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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). Italic represents shared alleles, and dashes indicate data not available. The smallest common region is between microsatellite markers 72GT2 and GT6. Markers MSFtri through GT1 lie within the \textit{SEPT9} gene. Markers MSFtri through GT1, indicated in bold type, lie within the \textit{SEPT9} gene.

**HNA patients possessing the shared haplotype have altered \textit{SEPT9} protein products**

Recent observations suggest that some \textit{SEPT9} isoforms are translationally regulated (19). To determine if \textit{SEPT9} translation was altered in HNA families containing the shared haplotype, we analyzed \textit{SEPT9} protein expression in LCL lysates from HNA patients, and unaffected family members using an antibody capable of recognizing \textit{SEPT9}i1-3 and i5/6 (immunogen noted in Fig. 1B). In control patients, the anti-\textit{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 \textit{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 \textit{SEPT9}i1, i2 or i3. We found that lysates from HNA patients sharing the founder haplotype express novel \textit{SEPT9} reactive protein bands at ~80 and ~100 kDa. A \textit{SEPT9}i1 specific antibody shows that one of the bands at 100 kDa and one at 74 kDa are \textit{SEPT9}i1 reactive. Actin expression serves as a loading control.

![Figure 2](https://academic.oup.com/hmg/article-abstract/18/7/1200/673112)
To determine whether 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 (Fig. 3A). No gross alterations in message were observed (Fig. 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 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.

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)
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 Fig. 7A) region of this exon and a reverse primer in the 5’ region (blue arrows Fig. 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 (Fig. 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 disrupts 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 kDa 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 kDa (20) and was not observed in LCLs from either shared haplotype or

(Fig. 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 has copy number changes within the SEPT9 gene (data not shown).

To verify the breakpoint between the duplicated regions, we designed forward (red arrows Fig. 5A) and reverse (blue arrows Fig. 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) (Fig. 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 (Fig. 5C).

Further analysis of all individuals in the founder haplotype pedigrees shows that the duplication does indeed segregate with the disease (Fig. 6; Supplementary Material, Fig. S1A and B). The penetrance of neurological attacks in these families is ~95%, which is consistent with previous reports (22). However, for many pedigrees recent clinical data were 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).
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. (19) 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. 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 suggest 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

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, 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.
cohort of HNA families (Hannibal et al., submitted for publication), 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 have 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, WA, USA). Genomic DNA was extracted as previously described (12). Short tandem repeat 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 CGH was performed using a custom oligonucleotide array consisting of 385,000 isothermal probes (NimbleGen Systems, Madison, WI, USA). 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/229 bp). Hybridizations were carried out as described previously (35) using a single normal male as reference (GM15724, Coriell, Camden, NJ, USA).

**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 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.

SUPPLEMENTARY MATERIAL
Supplementary Material is available at HMG online.

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Conflict of Interest statement. The authors declare that they have no conflicting interests.

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REFERENCES

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.


