VAMP1 Mutation Causes Dominant Hereditary Spastic Ataxia in Newfoundland Families

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Our group previously described and mapped to chromosomal region 12p13 a form of dominantly inherited hereditary spastic ataxia (HSA) in three large Newfoundland (Canada) families. This report identifies vesicle-associated membrane protein 1 (*VAMP1*), which encodes a critical protein for synaptic exocytosis, as the responsible gene. In total, 50 affected individuals from these families and three independent probands from Ontario (Canada) share the disease phenotype together with a disruptive *VAMP1* mutation that affects a critical donor site for the splicing of *VAMP1* isoforms. This mutation leads to the loss of the only *VAMP1* isoform (*VAMP1A*) expressed in the nervous system, thus highlighting an association between the well-studied VAMP1 and a neurological disorder. Given the variable phenotype seen in the affected individuals examined here, we believe that *VAMP1* should be tested for mutations in patients with either ataxia or spastic paraplegia.

Spastic ataxia 1 (SPAX1 [MIM 108600]) is a rare neurodegenerative disorder characterized by lower-limb spasticity and ataxia in the form of head jerks, ocular movement abnormalities, dysphagia, dysarthria, and gait disturbance[1,2] starting at the age of 10–20 years. Other clinical features are supranuclear gaze palsy, hyperreflexia, hypertonicity, dystonia, pes cavus, mild ptosis, and decreased vibration sense in the lower limbs. Symptom severity varies between individuals, but neither life span nor cognition is affected.[1,2] This disease resembles both spinocerebellar ataxias (SCAs [MIM 164400]) and hereditary spastic paraplegias (HSPs [MIM 182600]) but is part of a distinct group: hereditary spastic ataxias (HSAs). Previous reports describing the three Newfoundland (Canada) families affected by SPAX1 had indicated the existence of an ancestrally shared haplotype located in chromosomal region 12p13.[1,2]

A single neurologist (M.G.S.) diagnosed most of the family members (Figures S2, S3, and S4A, available online), and blood was collected for DNA analyses. More recently, Dr. Jog ascertained an additional family (Figure S4B) (with a Newfoundland origin [Figure S1]), and other neurologists (K.B., D.A.G., and S.G.) identified three single Ontario cases, all of whom presented with a similar SPAX1 phenotype. The collection of samples and genetic studies were approved by the relevant ethic committees, and informed consent was obtained from all subjects.

A mutation screening panel, composed of two affected individuals with the ancestral disease haplotype from two different families[2] (V-3 in Figure S2 and V-11 in Figure S3) and an unaffected control, was established. All coding exons and flanking intron regions of the 53 genes and predicted genes located in the 1.9 Mb disease haplotype were amplified by PCR. The PCR conditions and primer sets are available upon request. PCR products were sequenced at the McGill University and Génome Québec Innovation Centre (Montréal, Canada), and the sequences were analyzed with Mutation Surveyor v.3.0 (SoftGenetics). We searched for rare variants (those with less than 1% allele frequency) that were exclusively present in the two affected individuals of the mutation screening panel. Variants that were previously reported in dbSNP were excluded from further analysis. We determined the allele frequency of the remaining interesting variants by screening 169 Newfoundland population controls who were previously collected for a regional colorectal cancer study.[3]

A single variant of interest was observed in vesicle-associated membrane protein 1 (*VAMP1* [MIM 185880]) at position chr12: g.6574054T>G (Figure 1A). *VAMP1* has three annotated isoforms, *VAMP1A* (RefSeq accession number NM_014231.3), *VAMP1B* (RefSeq NM_016830.2), and *VAMP1D* (RefSeq NM_199245.1), that only differ by their last exon (Figure 1B). The primers amplifying *VAMP1* are given in Table S1. The mutation in *VAMP1A* and *VAMP1B* is c.340+2T>G, and the *VAMP1D* mutation is c.342T>G (p.Ser114Arg). In order to predict the impact of the variants, we used in silico programs (SIFT,[4] PolyPhen,[5] and

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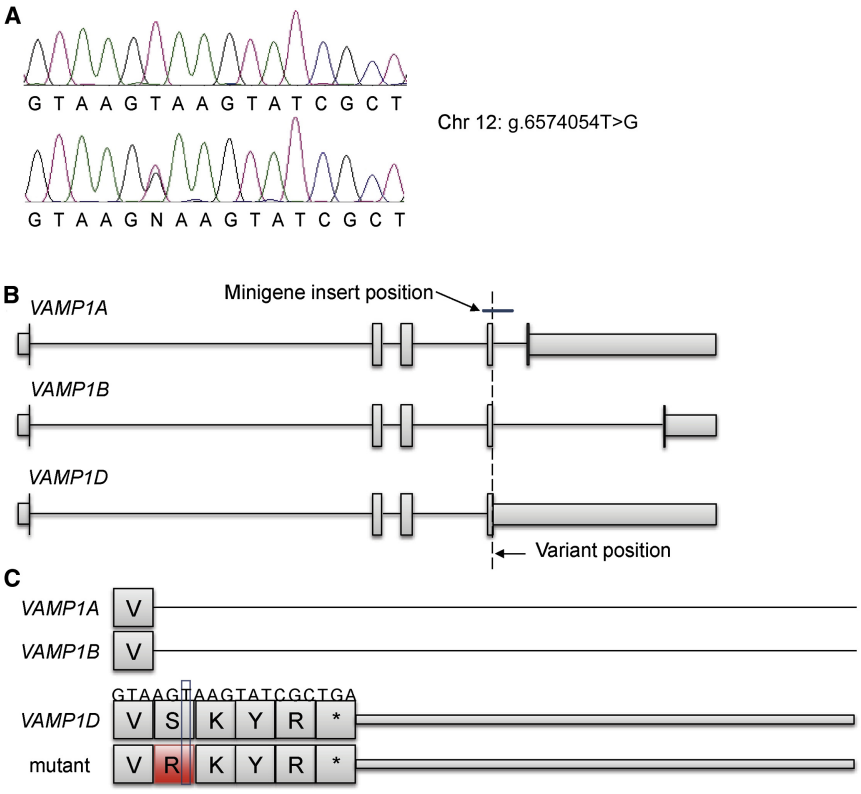


Figure 1. *VAMP1*, from the Gene to Its Encoded Protein

(A) A chromatogram of the variant chr12: g.6574054T>G from an affected individual is compared to that from a control.

(B) Representation of the three annotated *VAMP1* isoforms. The wider boxes are exons, the medium boxes are UTRs, and the lines are introns. The variant position and the insert localization are indicated.

(C) A zoomed-in view of the variant region, including the amino acid sequences of the annotated isoforms and the mutated sequence caused by the variant.

PANTHER[6]) to predict pathogenicity on protein structure and function and potential effect on RNA splicing (BDGP[7] and ESEfinder[8]). The p.Ser114Arg alteration is predicted to be damaging to the protein. The c.340+2T>G variant would disrupt mRNA splicing because it changes the GT splicing donor site to GG in both *VAMP1A* and *VAMP1B*.

Segregation of this particular variant was tested with genomic DNA from every participant, and the variant segregated with the disease in all four families, as well as in the three single cases from Ontario. The presence of this nucleotide mutation was also assessed in different populations with the use of three databases: 1000 Genomes (see Web Resources), the National Heart, Lung, and Blood Institute (NHLBI) Exome Variant Server (see Web Resources), and our in-house exome database composed of 708 individuals. In total, over 10,000 individuals from Newfoundland and various unrelated populations were looked at; none of them has the chr12: g.6574054T>G variant. Table 1 summarizes the genetic results of the 110 study participants, and each pedigree contains the information for each individual (Figures S2–S4). At the functional level, we used lymphoblastoid cell lines derived from patients in RT-PCR and immunoblot experiments to confirm the impact of the identified mutation at the RNA and protein levels. The expression of VAMP1 in these cells was, however, too low for either of these approaches to be informative, and unfortunately, no biopsy or autopsy material was available for conducting expression studies.

The mutation's impact on normal splicing was therefore assessed with two *VAMP1* minigenes (wild-type and mutant alleles) that were amplified from a patient's genomic DNA (V-3 in Figure S2); the two minigenes spanned from introns 3 to 4 (Figure 1B). Primer sequences and conditions are shown in Table S2. PCR products were separately inserted into pENTR/D-TOPO (Invitrogen) and transformed in One Shot TOP10 competent *E. Coli* (Invitrogen). The recombination reactions in pSPL3B_RFA, a modified pSPL3 vector (Invitrogen), were performed with Gateway LR Clonase II (Invitrogen). The cloning site of this vector is flanked by two constitutive exons from the rabbit beta-globin gene (RefSeq NM_001082260.2). The vectors were added to competent XL1 blue *E. coli* (Stratagene), and two independent colonies of each insert (mutant and wild-type) were selected for plasmid extraction with the QIAfilter Plasmid Midi Kit (QIAGEN). The sequences of the two inserts were confirmed before their transient expression in mammalian cells.

Lipofectamine 2000 (Invitrogen) was used for transfections in COS7 cells with 2 μg of DNA. Cells were harvested 48 hr after transfection and homogenized in TRIzol Reagent (Invitrogen) for RNA isolation. The cDNA was synthesized with the QuantiTect Reverse Transcription Kit (QIAGEN). GAPDH was used as a control for cDNA quantity. The RT-PCR of the studied variant was done with beta-globin primers: 5′ primer 5′-TCTGAGTCACCTG GACAACCT-3′ and 3′ primer 5′-ATCTCAGTGGTATTTGT GAGC-3′. PCR products were loaded on an agarose gel; bands were extracted and purified with the QIAquick Gel Extraction Kit (QIAGEN). The purified products were sent for sequencing, and the sequences were analyzed with SeqManII (DNASTAR).

Sequencing revealed that the wild-type allele (allele T) yielded a ~300 bp product that was predicted to arise from normal splicing. Also seen with the wild-type allele was a second product of ~400 bp. By opposition, the corresponding RT-PCR reactions prepared with the mutant

Table 1. Genetic Screening Results of the Families and Probands

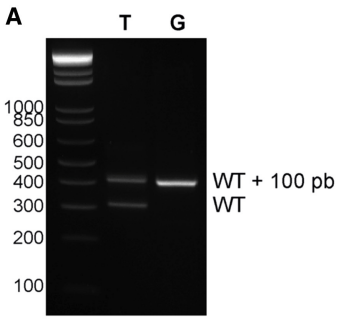
	Mutation Carrier			Mutation Noncarrier				
Family Name	Affected	Unaffected	Unknown	Affected	Unaffected	Married In	Unknown	Total
FPSAT6	6	0	3	0	1	0	0	10
Fam27	13	0	2	0	4	3	0	22
Fam71	10	0	0	0	13	1	0	24
Fam13	21	0	3	0	17	9	1	51
Probands	3	0	0	0	0	0	0	3
Total	53	0	8	0	35	13	1	110

allele cDNA (allele G at the variant position) only showed the ~400 bp product (Figure 2A). This observation fully supports our original prediction about the impact of the c.340+2T>G mutation on normal splicing. The ~400 bp fragment in cells expressing the wild-type minigene is most likely the consequence of the minigene system, which would have forced the use of any possible splicing sequence present. Moreover, the usual splice site of *VAMP1* in intron 4 has been shown to be a weak donor site.[9] The sequencing of the ~400 bp band revealed that another splicing donor site was used (Figure 2B). This downstream *VAMP1A* splice site is 99 bp after the variant position, and at the protein level, the resulting amino acid sequence due to aberrant splicing would be similar to that of VAMP1D because there is an in-frame stop codon before the splicing site. Furthermore, the mutant cDNA would produce a VAMP1D protein containing a missense alteration: p.Ser114Arg (Figure 1C).

The neuronal VAMPs are involved in the synaptic vesicle cycle at the presynaptic nerve terminal. They are anchored in the vesicle membrane by their C-terminal domain and interact with synaptophysin prior to docking of the vesicle. When docking happens, they bind the complex SNAP-25 and syntaxin to form the synaptic core complex, starting the cascade of protein-protein interactions leading to neurotransmitter exocytosis. Several clostridial toxins, namely tetanus toxin and botulinum toxins B, D, F, and G, are known to cleave VAMP proteins. These toxins block the release of neurotransmitters either in the CNS or in the peripheral nervous system, arguing that VAMPs are essential for exocytosis.[10,11]

VAMP proteins have been extensively studied with rat (*Rattus norvegicus*) models, although the electric ray (*Torpedo californica*) was the first organism in which *VAMP1* (*synaptobrevin 1* or *SYB_TORCA*) was reported.[12,13] The first discovered synaptobrevin protein (Vamp1) was reported to be expressed in the nervous system and endocrine cells, and it was also found that the rat genome contains two different genes encoding two similar proteins, *Vamp1* and *Vamp2*;[12–14] both are expressed in the nervous system but at distinct localizations. *Vamp1* is expressed in the rat cerebellum, a structure known to be involved in ataxias in humans.[15] The protein is also present in the rat brainstem and spinal cord, more specifically in the nuclei controlling eye movements, tongue movements, swallowing, and limb movements.[16–18] All these CNS structures are consistent with some of the SPAX1 symptoms.

A few years after the original report describing *Vamp1*, the use of an alternative splice site was shown to underlie a distinct isoform that was henceforth named *Vamp1b*. This second isoform is exclusively expressed in nonneuronal rat



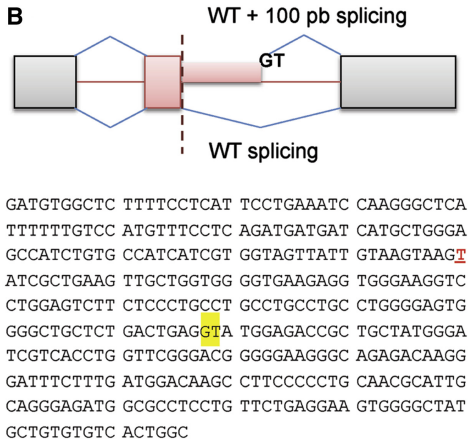


Figure 2. Testing Splicing with RNA from Transfected COS7 Cells
(A) Agarose-gel result of RT-PCR on RNA extracted from COS7 cells transfected with a *VAMP1* minigene. The allele present in the insert is indicated above the corresponding lane.
(B) Representation of the plasmid portion relevant for RT-PCR. The beta-globin exons are in gray, the splicing events are represented by blue lines, and the variant position is indicated by the red dashed line. The insert, in red, has introns (lines) and an exon (wider box). The medium-sized box represents the nucleotides added by the abnormal splicing. The presented sequence represents the sequence of the insert; the mutated T is highlighted in red, and the alternatively used donor site is highlighted in yellow.

tissues, such as the kidneys, spleen, liver, parotid, and pancreas.[9] The isoform *Vamp1b* region that diverged from the original isoform, now termed *Vamp1a*, was in the C-terminal of the protein. The authors suggested that the splicing was tissue specific and that the normal splicing was disrupted as a result of a weak 5′ donor splicing motif.[9] In this last isoform, exon 4 continues, leading to a product ~400 bp larger. Moreover, 3 years ago, another group found a *Vamp1* isoform, termed *Vamp1nv*, specific to the rat's cochlear nucleus; again, it diverges only in the C-terminal of the protein.[19]

In 1999, a report by Berglund[20] and colleagues identified six distinct *VAMP1* isoforms, but only three of these are presently listed in the human genome (GRCh37/hg19). The *VAMP1A* (RefSeq NM_014231.3) isoform is mostly expressed in the nervous system,[20] and the protein sequence encoded by this isoform is similar to the electric ray SYB_TORCA and rat Vamp1a orthologs. *VAMP1B* (RefSeq NM_016830.2) is an isoform with a broad tissue expression (e.g., T cells, neutrophils, fibroblasts, liver cells, endothelial cells, and more) but is noticeably not expressed in the human brain.[17] In fact, the expression of VAMP1A and VAMP1B is believed to be, to a certain extent, mutually exclusive.[17] In humans, the *VAMP1A* and *VAMP1B* isoforms encompass the same first four exons but the following and last exon, which carries a mitochondrial targeting signal in *VAMP1B*, is distinct between these two isoforms.[21,22] The third *VAMP1* isoform is *VAMP1D* (RefSeq NM_199245.1), which is almost identical to the rat *Vamp1b* and is therefore probably not expressed in the nervous system. In this third isoform, the translation of the fourth exon continues until it reaches a stop codon 12 nucleotides downstream (c.354). The normal donor site located at the end of exon 4 (c.340+2), which is critical for the differential isoform splicing events, is the donor site mutated in the SPAX1 patients. Therefore, the mRNA normal splicing of both *VAMP1A* and *VAMP1B* is likely to be affected.

Using RNA-Seq data from Illumina's Human BodyMap 2.0 (see Web Resources), we were able to confirm the presence of *VAMP1A* mRNA in the Brain and Brain_R libraries by using the Scripture and Cufflinks programs. A *VAMP1B* mRNA sequence was shown only once in the Brain library. As for *VAMP1D*, it never appeared to be expressed in the brain. Consequently, we predict that the mutation in patients leads to alternative splicing with the formation of an abnormal inactive isoform in neurons and subsequently results in haploinsufficiency. Therefore, there would be less neurotransmitter exocytosis in specific regions of the brain, causing the symptoms of SPAX1. Without autopsy material, it is not possible to determine whether the p.Ser114Arg VAMP1D is aberrantly expressed in the brain and thereby possibly leads to neuronal damage. However, given that this isoform is normally expressed only in nonneuronal tissues, it seems unlikely that it will lead to the HSA phenotype.

There is a spontaneous Vamp1-null mouse model called lethal wasting (*lew*), in which a mutation causes the truncation of half of the protein; hence, no Vamp1 is produced. The phenotype in homozygous mice is mostly neurological defects, and the animals die at postnatal day 15.[23] In the mouse, *Vamp1* appears to encode a protein that is essential for the function of neuromuscular junction, specifically for Ca^{2+} sensitivity.[24] Other synapses were not studied, but one could speculate that the role of VAMP1 in the CNS is likely to be similar. *Vamp1*-heterozygous mice appear normal; however, a detailed assessment of the impact on the brain was not done (W. Lin, personal communication).

We have identified a *VAMP1* variant that segregates in four HSA-affected families from Newfoundland and that is present in three additional probands. This variant is absent from 169 Newfoundland population controls (the affected families are from Newfoundland), as well as every other population looked at. Using a minigene, we established that the variant has an effect on the splicing of the RNA primarily affecting a neuron-specific isoform. We suspect that the amount of this neuron-specific VAMP1 isoform is reduced by half and that this leads to the neurological deficiencies observed clinically. Because this variant is the only rare and heterozygous one found in the 1.9 Mb disease-haplotype region and because it is putatively damaging, the odds of its being disease causing are high. Nonetheless, experiments with patients' tissues or, perhaps, animal models will be needed for establishing the disease mechanism and for further validating this variant as the real causative one.

Patients with HSA, HSP, or ataxia and who have ancestral links in Newfoundland should be tested for mutations in this gene. Indeed, VAMP1 is part of the membrane-trafficking and axonal transport family of proteins,[10] and proteins with these roles have been linked to both dominant ataxias[25] and HSPs,[26] which encompass the HSA phenotype. Thus, beyond the similarity of phenotypes, the roles of VAMP1 make it a relevant candidate for all three families of disorders. It also remains to be determined whether mutations in this gene might be found in patients outside of this Newfoundland founder effect.

Supplemental Data

Supplemental Data include four figures and two tables and can be found with this article online at http://www.cell.com/AJHG.

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Web Resources

The URLs for data presented herein are as follows:

1000 Genomes, http://www.1000genomes.org/
dbSNP, http://www.ncbi.nlm.nih.gov/snp/
Illumina's Body Map 2.0 Transcriptome, http://www.ebi.ac.uk/arrayexpress/browse.html?keywords=%20E-MTAB-513
NHLBI Exome Sequencing Project Exome Variant Server, http://evs.gs.washington.edu/EVS/
Online Mendelian Inheritance in Man (OMIM), http://www.omim.org
UCSC Genome Browser, http://genome.ucsc.edu/

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