RESEARCH ARTICLE

REVISED Novel somatic single nucleotide variants within the RNA binding protein hnRNP A1 in multiple sclerosis patients [v2; ref status: indexed, http://f1000r.es/4dh]

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Abstract

Some somatic single nucleotide variants (SNVs) are thought to be pathogenic, leading to neurological disease. We hypothesized that heterogeneous nuclear ribonuclear protein A1 (hnRNP A1), an autoantigen associated with multiple sclerosis (MS) would contain SNVs. MS patients develop antibodies to hnRNP $A1^{293-304}$, an epitope within the M9 domain ($AA^{268-305}$) of hnRNP A1. M9 is hnRNP A1's nucleocytoplasmic transport domain, which binds transportin-1 (TPNO-1) and allows for hnRNP A1's transport into and out of the nucleus. Genomic DNA sequencing of M9 revealed nine novel SNVs that resulted in an amino acid substitution in MS patients that were not present in controls. SNVs occurred within the TPNO-1 binding domain (hnRNP $A1^{268-289}$) and the MS IgG epitope (hnRNP $A1^{293-304}$), within M9. In contrast to the nuclear localization of wild type (WT) hnRNP A1, mutant hnRNP A1 mis-localized to the cytoplasm, co-localized with stress granules and caused cellular apoptosis. Whilst WT hnRNP A1 bound TPNO-1, mutant hnRNP A1 showed reduced TPNO-1 binding. These data suggest SNVs in hnRNP A1 might contribute to pathogenesis of MS.

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Invited Referee Responses

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version 1 published 20 Jun 2014	report 1	report 1	report 1
version 2 published 18 Sep 2014 REVISED			

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Latest Comments

No Comments Yet

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Competing interests: Drs. Michael Levin and Sangmin Lee have a United States patent pending titled “Biomarker for neurodegeneration in neurological disease”.

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REVISED **Amendments from Version 1**

We appreciate the reviewer's comments and have addressed them in the revised manuscript. None of the reviewers required further experiments. The reviewers requested we include data indicating that SNVs may be the result of oxidative stress or clonal expansion of T-cells. They also suggested that SNVs in CNS targets other than neurons might be important in the pathogenesis of MS and that future studies should include more genes and control groups. All of these suggestions were specifically addressed in the discussion.

See referee reports

Introduction

Multiple sclerosis (MS) is the most common autoimmune disease of the central nervous system (CNS) in humans, whose pathogenesis remains unknown. A number of genetic and immune studies indicate dysregulated immune responses as contributors to the pathogenesis of MS[1–7]. Genetic analyses show an association of MS with major histocompatibility complex (MHC) Class II human leukocyte antigen (HLA)-DRB-1 and single nucleotide polymorphisms (SNPs) related to immune function[1,2,8]. Both Th1/Th17 $CD4^+$ T-lymphocytes and immunoglobulins appear to have a causative role[1,2,9]. Immunoglobulin G (IgG) responses to myelin and non-myelin targets have differentiated some MS patients from healthy controls[9–11]. Non-myelin antigens that are targets for immunoglobulins isolated from MS patients include neurofilaments, axonal neurofascin and RNA binding proteins, including heterogeneous nuclear ribonucleoprotein A1 (hnRNP A1)[9,12–16].

Recently, mutations in RNA binding proteins have been shown to cause neurological disease[17–21]. For example, a mutation (p.D263V) in the prion-like domain (PrLD) of hnRNP A1 has been shown to cause familial amyotrophic lateral sclerosis (ALS)[22]. In addition to inherited mutations, somatic variants have also been shown to cause neurological disease[23]. hnRNP A1 performs a number of critical cellular functions related to transcription, nucleocytoplasmic transport of mRNA and translation[24,25]. In addition to the PrLD, other important functional domains in hnRNP A1 include two RNA binding domains (RBDs) and M9, its nucleocytoplasmic shuttling domain[22]. M9 binds its nuclear receptor, transportin-1 (TPNO-1, also known as karyopherin β2) and the hnRNP A1:TPNO-1 complex is transported into and out of the nucleus[3,9,16,26,27].

Our lab has performed extensive studies on the role of autoimmunity to hnRNP A1 in MS and human T-lymphotropic virus type 1 (HTLV-1) associated myelopathy/tropical spastic paraparesis (HAM/TSP), a viral-induced model and clinical mimic of MS[3,28–30]. Initially, we discovered that HAM/TSP patients develop antibodies to hnRNP A1 that cross-react with HTLV-1-tax, indicative of molecular mimicry[29,31]. Next, the epitope of the HAM/TSP IgG response ($AA^{293\text{-}304}$) was localized to M9 ($AA^{268\text{-}305}$)[32]. M9 is a bipartite phenylalanine-tyrosine nuclear localization sequence (PY-NLS) that requires binding to TPNO-1 for hnRNP A1 to shuttle between the nucleus and cytoplasm[16,31] Because of the similarities between MS and HAM/TSP, we hypothesized that MS patients would also develop antibodies to hnRNP A1. In fact, antibodies isolated from MS patients, in contrast to healthy controls and Alzheimer's patients, were also found to immunoreact with the identical hnRNP-A1-M9 epitope ($AA^{293\text{-}304}$)[16]. Subsequent studies indicated that the IgG was biologically active and potentially pathogenic. For example, mono-specific antibodies to hnRNP A1 isolated from patients caused decreased neuronal firing using neuronal patch clamp in rat brain sections[31,33]. Further, neurons exposed to anti-hnRNP A1-$M9^{293\text{-}304}$ specific antibodies resulted in neurodegeneration and neuronal death[16,34]. The anti-hnRNP A1-$M9^{293\text{-}304}$ specific antibodies also caused changes in neuronal RNA expression that correlate with the clinical phenotype of MS and HAM/TSP patients (ie. spastic paraparesis), which was subsequently confirmed in neurons isolated from the brains of MS patients[16]. Additional studies showed that anti-hnRNP A1-$M9^{293\text{-}304}$ specific antibodies entered neurons via clathrin-mediated endocytosis and caused apoptosis in a neuronal cell line[34]. Anti-hnRNP A1-$M9^{293\text{-}304}$ specific antibodies also caused a redistribution of hnRNP A1 in neurons from nuclear to an equal distribution of nuclear and cytoplasmic localization, suggesting the antibodies interfered with M9, which is required for hnRNP A1s nuclear import[34]. Considering: (1) the role of hnRNP A1 in cellular function; (2) variant forms of hnRNP A1 cause neurodegenerative disease, and (3) hnRNP A1 is an autoimmune target in MS patients, we hypothesized that MS patients would contain novel genomic DNA single nucleotide variants (SNVs) in hnRNP A1-M9, which when expressed, would alter cellular function and contribute to cell death.

Methods

Patients

All blood samples were collected according to the approved Institutional Review Board protocols (Veterans Affairs Medical Center - Memphis, Study #317164, University of Tennessee Health Science Center, Study #98-06618-FB) with patient consent. The diagnosis of MS was made using published criteria (Supplement 1, see Results)[35].

Preparation of human peripheral blood monocytes (PBMCs) and isolation of genomic DNA

Human PBMCs were isolated from fresh blood by Ficoll-Paque gradient centrifugation and washed with PBS. Genomic DNA was isolated from PBMCs using the QIAmp blood kit (Quiagen Inc., Chatsworth, CA, U.S.A.) according to manufacturer's protocol. All DNA samples were quantified using Nanodrop (Quawell) and restriction enzyme digestion methods.

PCR primers

Specific oligonucleotides were designed from the published genomic DNA sequence of the human hnRNP A1 gene. The upstream primer 5′- CAGATAAAGGC CCTCTTTCCC -3′ (3080–3100) and the downstream primer 5′- CTCAGCTACATTAGGGTTATTGGG -3′ (3667–3690) flank a 611 bp region of the human hnRNP A1 genomic DNA containing exon 8 and exon 9.

PCR amplification and subcloning

One microgram of genomic DNA was amplified in a reaction mixture containing the primers and KOD Hot Start DNA polymerase (Novagen). Use of this DNA polymerase has a mutation frequency of 0.10%[36]. Before adding enzyme, the reaction mixture was heated at 95°C for 2 minutes. Amplification was carried out for 35 cycles of denaturation at 95°C for 20 s, annealing at 57°C for 10 s, and extension at 72°C for 15 s, followed by terminal elongation at 70°C for 20 s. The resulting PCR product was cloned into the pCR2.1-TOPO

blunt vector (Invitrogen), yielding pCR2.1-TOPO-Blunt-hnRNP A1-611 bp, and *E. coli* TOP10 was transformed with this plasmid. Purified plasmid was digested with *EcoRI* yielding either one band (no insert) or two bands, 3.9 kbp (plasmid) and 611 bp (insert). Digests were subjected to electrophoresis on 1.5% agarose gel and visualized with the Gel Logic 200 Imaging System (Kodak). Clones that contained the 611 bp insert were sequenced (Supplement 3).

DNA sequencing and sequence analysis

Tue pCR2.1-TOPO-Blunt-hnRNP A1 611 bp clones were subjected directly to automated DNA sequencing (ABI 3130 X L) at the University of Tennessee Health Sciences Center Molecular Resource Center. Electropherograms were obtained and sequence quality was analyzed by Sequence Analysis Software (ABI). Sequence alignment was carried out by Nucleotide BLAST (National Center for Biotechnology Information Called genomic DNA sequences were compared to mutations (SNVs, SNPs) listed in four different public databases: (1) Exome variant server (ESV): http://evs.gs.washington.edu/EVS/, (2) Catalogue of somatic mutations in cancer (COSMIC): http://cancer.sanger.ac.uk/cancergenome/projects/cosmic/, (3) 1000 genomes; a deep catalog of human genetic variation: http://www.1000genomes.org, (4) NCBI dbSNP: (http://www.ncbi.nlm.nih.gov/snp/).

Cloning and expression of hnRNP-A1

cDNA encoding the entire sequence of hnRNP A1 (WT) was cloned into the expression vector pTriEx™5 Ek/LIC vector (Novagen) and transfected into SK-N-SH cells, a neuroblastoma cell line (ATCC - American Type Culture Collection). The amplified open reading frame (ORF) of hnRNP A1 was subcloned into *Bam* HI and *Hin*d III sites of modified pGEX-6p-1 vector to create recombinant *E. coli* expression vectors for gluthathione S-transferase (GST) full down assay.

Primers and site-directed mutagenesis

The primers for mutagenesis by PCR were designed basically according to the manufacturer (QuikChange™ II XL Site-Directed Mutagenesis kit; Agilent Technologies, CA). Briefly, each pair of primers contained a primer-primer complementary (overlapping) sequence at the 3′- and 5′-terminus. The designed primers were used for mutagenesis of the target residues F273L, M276L and F281L in hnRNP A1. The primers for each of the variants were: (1) p.F273L - forward: CAG TCT TCA AAT **CTT** GGA CCC ATG AAG GGA GG, reverse: CCT CCC TTC **AGG** GGT CCA AAA TTT GAA GAC TG; (2) p.M276L - forward: CAG TCT TCA AAT TTT GGA CCC **CTG** AAG GGA G, reverse: CCT CCC TTC ATG GGT CCA **AGA** TTT GAA GAC TG; (3) p.F281L - forward: C ATG AAG GGA GGA AAT **CTT** GGA GGC AGA AGC TC, reverse: GA GCT TCT GCC TCC **AAG** ATT TCC TCC CTT CAT G. All variant sites were located in hnRNPA1-M9 and both forward and reverse primers shared the region in question. The melting temperature (T_m) was calculated using the formula provided by the manufacturer Agilent Technologies: $T_m = 81.5+0.41(\%GC)-675/N-\%\ mismatch.$ Here, *N* is the primer length in bases. All the primers were synthesized by Genelink (Hawthorne, NY). Mutagenic reaction was performed in 50 µl of PCR mix containing 10 ng of pTriEx-5 Ek/LIC-hnRNP A1(WT) or pGEX-6p-1-hnRNP A1(WT) as template, 200 nM primer and 2.5 U Pfu DNA polymerase. The PCR temperature profile was: an initial denaturation at 95°C for 1min, followed by 18 cycles with each at 95°C for 50 sec, 60°C for 50 sec and 68°C for 1 kb/min, and a final extension at 68°C for 7 min. The PCR products of Site-Directed Mutagenesis were transformed into *E. coli* XL10-Gold competent cells and isolated using Qiagen miniprep kits (Qiagen, Germany).

Transfection

DNA complexes prepared using a DNA (µg) to Lipofectamine® 2000 (µl) ratio of 1:2.5 for SK-N-SH cell line. For hnRNP A1 relocalization experiments, the human hnRNP A1 (WT or variant) cDNA was transfected into SK-N-SH cells (70–80% confluence) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. After 5 hours incubation, the transfection mixture was removed from each well and replaced with DMEM containing 10% FBS. Fresh medium was conditioned for 24 h before relocalization analysis of hnRNP A1 by immunocytochemistry.

Immunocytochemistry

SK-N-SH Cells (ATCC HTB-11) were grown on poly-L-lysine-coated cover slips and were transfected using Lipofectamine 2000. Cells were then rinsed with PBS, fixed with 4% paraformaldehyde, permeabilized with cold acetone, and blocked in PBS containing 5% BSA. Primary antibodies used were: rabbit anti-TDP-43 (1:1000, Millipore, catalog #ABN271), rabbit anti-active caspase-3 (1:50, Millipore, catalog #AB3623), rabbit anti-Neuron specific beta III tubulin (NTB3) (1:1000, Abcam, catalog #ab18207) and biotinylated mouse anti-strep-Tag II (1:1000, GenScript, catalog #A01737). Secondary antibodies were: Texas Red conjugated goat anti-rabbit IgG (1:300, Vector, catalog #TI-5000 and FITC conjugated strepavidin (1:300, Vector, catalog #SA-5001). Primary antibodies were diluted in blocking solution incubated with each coverslip for overnight at 4°C. Cells were then washed with PBS and incubated in secondary antibody for 1 hr. Cells were then washed with PBS and mounted in Prolong-Gold anti-fade reagent with DAPI (Invitrogen).

GST pull-down assay

SK-N-SH cells were cultured in Dulbecco's Modified Eagle's medium (BD Biosciences) supplemented with 10% fetal bovine serum, 100 U/mL penicillin G and 100 µg/mL streptomycin, at 37°C under 5% CO_2. Cells were harvested and lysed with CytoBuster™ Protein Extraction Reagent (Millipore), containing inhibitor cocktail, homogenized for a few seconds with a handheld homogenizer and spun at 16,000 × g for 5 minutes. Supernatants were used for GST-pull down assays. Glutathione-Sepharose 4B beads coupled with GST-hnRNP A1 (WT or variant), which includes the Transportin 1-binding domain, were incubated for 1 h at 4°C with 600 µL of the cell lysates in CytoBuster™ Protein Extraction Reagent and protease inhibitors. After washing the beads three times with 600 µL of 10 mM PBS (10 mM Na_2HPO_4, 140 mM NaCl, 2.7 mM KCl, 1.8 mM KH_2PO_4, pH 7.4) and protease inhibitors, proteins bound to the beads were analyzed by 8–16% SDS-PAGE followed by immunoblotting with rabbit polyclonal GST antibody (1:1000, Millipore, catalog #06-332), mouse monoclonal Transportin 1 antibody (1:1000, Millipore, catalog #05-1515) and mouse monoclonal TDP-43 antibody(1:1000, Millipore, catalog #MABN45). The immunoreactive bands were visualized using enhanced chemiluminescence.

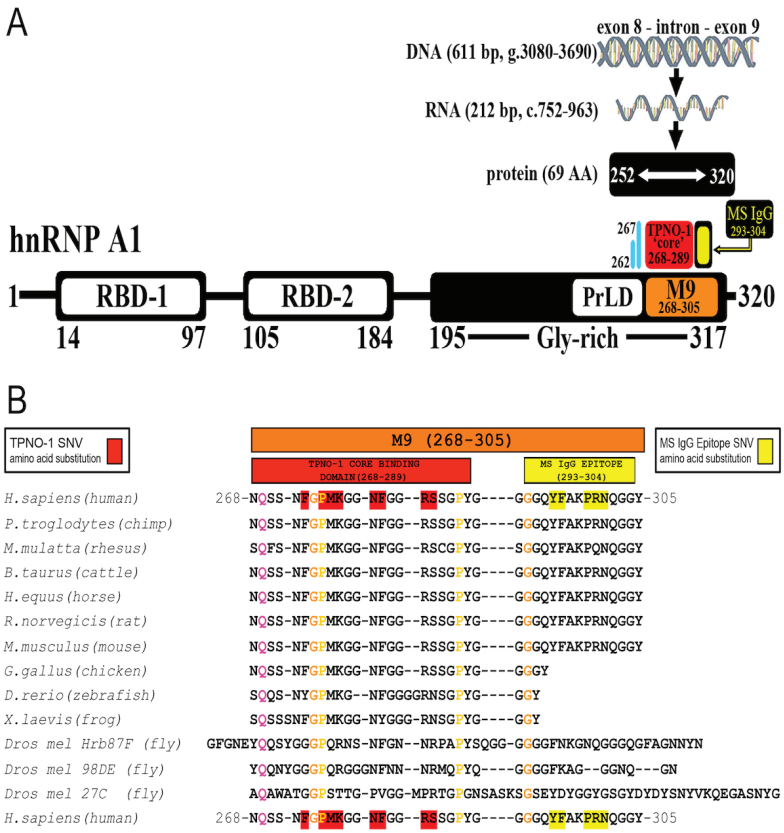

Figure 1. Functional domains and sequence alignment of hnRNP A1. A: Domain schematic of hnRNP A1. The sequence shown is isoform A (NP_002127). Isoform B contains a 52 amino acid insert following AA^{251}, resulting in a 372 amino acid protein (not shown). The RNA binding domains (RBD 1 and 2) are contained within the N-terminal half of hnRNP A1. The prion-like domain (PrLD, $AA^{233-267}$), M9 ($AA^{268-305}$) and the C-terminus ($AA^{306-320}$) are shown. M9 (orange) contains the 'core' transportin-1 binding domain (TPNO-1, $AA^{268-289}$, red) and the MS IgG epitope ($AA^{293-304}$, yellow). Some literature indicates that the PrLD and TPNO-1 binding domain may overlap, such that the PrLD region includes $AA^{233-272}$ and the TPNO-1 binding domain includes $AA^{263-289}$, with the a resulting overlap of $AA^{263-272}$ (Supplement 2). The amplicon included DNA from exons 8 and 9 with the intervening intron. The expressed protein included the PrLD that contained ALS-associated mutations (p.D262V (familial), p.N267S (sporadic)(blue)), as well as M9 and the C-terminus of hnRNP A1. (bp base pair). **B**: Sequence alignment of hnRNP A1-M9. Human sequences are 100% conserved in mammals, except for *M. mulatta* (rhesus). There is also high sequence conservation between orthologs (mammals, non-mammalian vertebrates (*G. gallus* - chicken, *D. rerio* - zebrafish, *X. laevis* - frog) and invertebrates (*D. melanogaster* - fruit fly). SNVs resulting in amino acid substitutions in the TPNO-1 core domain are highlighted in red boxes. SNVs resulting in amino acid substitutions in the MS IgG epitope are highlighted in yellow boxes. Colored amino acids are present for clarity identifying conserved sequences through all species. Black lines (gaps) are inserted between residues so that similar or identical amino acids are aligned in each column. (http://www.bioinformatics.org/strap/).

Results

Novel somatic DNA SNVs are contained within the TPNO-1 binding domain and MS IgG epitope of hnRNP A1-M9 in MS patients

We sequenced a 611 bp region of hnRNP A1 genomic DNA inclusive of exons 8 and 9 with intervening introns (NP_002127.1, Chromosome 12q13.1, DNA g.3080-3690, RNA (cDNA) c.752-963, protein $AA^{252-320}$) (Figure 1A) isolated from the PBMCs of patients with MS: relapsing remitting MS (RRMS, n=5), secondary progressive (SPMS, n=5) and primary progressive MS (PPMS, n=4) and healthy controls (HC, n=6) (Supplement 1). The expressed sequence included: the C-terminal of the PrLD ($AA^{252-267}$), M9 ($AA^{268-305}$) and the residual C-terminus of hnRNP A1 ($AA^{306-320}$) (Figure 1A). This region also includes the 'core' TPNO-1 binding domain ($AA^{268-289}$) and the MS IgG epitope ($AA^{293-304}$). Some literature indicates that the PrLD and TPNO-1 binding domain may overlap, such that the PrLD region includes $AA^{233-272}$ and the TPNO-1 binding domain includes $AA^{263-289}$, with the a resulting overlap of $AA^{263-272}$ (Supplement 2)[22,26]. In addition, the previously reported mutations (p.D262V (familial), p.N267S (sporadic)) that cause ALS are also contained within the target sequence (Figure 1A, Supplement 2)[22]. A small percentage of clones from each individual contained genomic DNA SNVs, indicative of these being somatic SNVs derived from a small percentage or subset of PBMC. SNVs were compared to those found in four different databases (see Methods and Supplement 2).

Of the six HCs, zero SNVs resulted in an amino acid substitution within the TPNO-1 binding domain, MS IgG epitope or M9 from the 481 clones that were sequenced (Table 1, Supplement 2, Supplement 3). One individual had a likely benign variant which did not result in a change in the associated amino acid (c.900A>G, p.R300R) (Supplement 2), and three others had SNVs in the C-terminal region

Table 1. hnRNP A1 single nucleotide variants that resulted in an amino acid substitution. HC: Healthy Control. RRMS: Relapsing Remitting Multiple Sclerosis. SPMS: Secondary Progressive Multiple Sclerosis. PPMS: Primary Progressive Multiple Sclerosis. TPNO-1: Transportin 1. PRLD: Prion-like Domain. AA: aminoacid. SNV: single nucleotide variant.

EXON>	EXON 8>																						<EXON 9	<EXON
DOMAIN>	251<----------PrLD----------> 267				268<----------M9----------> 305													306<----------C-terminal----------> 320						<DOMAIN
DOMAIN>	(233)				268<----------TPNO-1 Binding Domain----------> 289								293<----------MS IgG Epitope----------> 304											<DOMAIN
AA SNV site>	252	259	263	265	273	275	276	277	280	281	284	285	295	296	299	300	301	308	313	314	317	318	319	320	<AA SNV site
PPMS-1				793 A>G		823 C>T															950 G>A				PPMS-1
				N265D		P275S															G317D				
PPMS-2								831 G>T																	PPMS-2
								K277N																	
PPMS-3				793 A>G							850 A>G														PPMS-3
				N265D							R284G														
PPMS-4					817 T>C					841 T>C		853 A>G					901 A>G	922 T>G							PPMS-4
					F273L					F281L		S285G					N301D	S308P							
SPMS-1			787 T>C												896 C>T	898 C>T				941 A>G				958 T>C	SPMS-1
			F263L												P299L	R300S				Y314C				F320L	
SPMS-2																									SPMS-2
SPMS-3					817 T>G																				SPMS-3
					F273L																				
SPMS-4	755 G>A												884 A>G	886 T>C			902 A>G								SPMS-4
	S252N												Y295C	F296L			N301S								
SPMS-5																									SPMS-5
RRMS-1																			937 A>G				955 A>G		RRMS-1
																			S313G				R319G		
RRMS-2							826 A>C		839 A>G											940 T>C					RRMS-2
							M276L		N280S											Y314H					
RRMS-3																									RRMS-3
RRMS-4																									RRMS-4
RRMS-5		775 A>G																							RRMS-5
		S259G																							
HC-1																									HC-1
HC-2																									HC-2
HC-3																									HC-3
HC-4																		922 T>C							HC-4
																		S308P							
HC-5																						952 A>G			HC-5
																						R318G			
HC-6																					949 G>A				HC-6
																					G317S				
DOMAIN>					268<----------TPNO-1 Binding Domain----------> 289								293<----------MS IgG Epitope----------> 304											<DOMAIN
DOMAIN>	233<----------PrLD----------> 267				268<----------M9----------> 305													306<----------C-terminal----------> 320						<DOMAIN
EXON>	EXON 8>																						<EXON 9	<EXON

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(c.922T>C, p.S308P; c.949G>A, p.G317S; c.952A>G, p.R318G), which altered the amino acid sequence (Table 1, Supplement 2, Supplement 3). The SNV at AA[308] was previously reported and not associated with disease (http://www.ncbi.nlm.nih.gov/snp). These data, in which SNVs in hnRNP A1 are a rare event, are consistent with the finding in sporadic (1 of 305) and familial ALS (1 of 212) patients where most did not have mutations by whole exome sequencing[22].

Of the five RRMS patients in which 358 clones were sequenced, one patient had two novel SNVs contained within the TPNO-1 binding domain that resulted in an amino acid substitution (c.826A>C, p.M276L; c.839A>G, p.N280S) (Table 1, Supplement 2, Supplement 3, Data availability). None of the other RRMS patients had changes within the TPNO-1 binding domain or M9. Other SNVs that resulted in an amino acid substitution included those within the C-terminal region (c.937A>G, p.S313G; c.940T>C, p.Y314H; c.955A>G, p.R319G) and one within the PrLD (c.775A>G, p.S259G). These SNVs are also novel (Table 1, Supplement 2, Data availability). There was a single SNV (c.963A>G) that did not alter the stop codon amino acid sequence (Supplement 2).

Of the five SPMS patients, one had a novel SNV that resulted in an amino acid substitution in the 'core' TPNO-1 binding domain (c.817T>G, p.F273L). Although there was a somatic SNV contained within this codon in the COSMIC database (in patients with cancer), its SNV and amino acid change were different (c.818T>G, p.F273C). A second patient had a novel SNV contained within the PrLD (c.755G>A, p.S252N), which also aligned with a somatic SNV in the COSMIC database (c.755G>T, p.S252I), but yielded different amino acids. A third patient had an SNV within the PrLD (c.787T>C, p.F263L), which also aligned with a somatic SNV contained within this codon in the COSMIC database (c.789T>G, p.F263L). In contrast to HC, RRMS or PPMS, novel SNVs that resulted in an amino acid substitution in SPMS predominated within the MS IgG binding epitope of M9 (c.884A>G, p.Y295C; c.886T>C, p.F296L; c896C>T, p.P299L; c.898C>T, p.R300S; c.902A>G, p.N301S) (Table 1, Supplement 2, Supplement 3, Data availability). Overall, of the 355 clones that were sequenced from the five SPMS patients, 8 (2.25%) were contained within the PrLD and M9 domains of which 6 were within M9, and 5 were exclusive to the MS IgG epitope (Table 1, Supplement 2, Supplement 3). Like HC and RRMS, there were several SNVs contained in the C-terminal region of hnRNP A1 (c.941A>G, p.Y314C; c.958T>C, p.F320L) (Table 1, Supplement 2, Supplement 3).

In contrast to HC, all four PPMS patients, had novel somatic SNVs that resulted in an amino acid substitution and were contained within the 'core' TPNO-1 binding domain of hnRNP A1 as follows: patient 1 (c.823C>T, p.P275S), patient 2 (c.831G>T, p.K277N), patient 3 (c.850A>G, p.R284G), and patient 4 (c.817T>C, p.F273L; c.841T>C, p.F281L; c.853A>G, p.S285G) (Table 1, Supplement 2, Data availability). Patients 1 and 3 each had a novel SNV within the PrLD region (c.793A>G, p.N265D). Thus, of the 317 clones that were sequenced, 2.84% were contained within the M9 or PrLD domains, two-thirds of which were exclusive to the TPNO-1 binding domain (Supplement 3). Other SNVs were contained within the MS IgG binding epitope (c.901A>G, p.N301D) or the C-terminal region (c.922T>G, p.S308P; c.950G>A, p.G317D) (Table 1, Supplement 2, Supplement 3, Data availability). Only the c.817T>C, p.F273L SNV aligned with a somatic SNV within the same codon in the COSMIC database (c.818T>G, p.F273C), but again, both the SNV and amino acid substitution differed.

The TPNO-1 binding domain is highly conserved within mammals and evolutionarily conserved between species (Figure 1B). Specifically, the TPNO-1 binding domain is 100% conserved in mammals, except for five mutations contained only within the rhesus monkey (*Macaca mulatta*) (only one of which overlapped with the SNVs we discovered (AA[300]) (Figure 1B)). Further, amino acid sequences were highly conserved between species, as shown by the orthologs between mammals, bird (*Gallus gallus*), fish (*Danio rerio*) and frog (*Xenopus laevis*) as well as with the fruit fly (*Drosophila melanogaster*) (Figure 1B). Taken together, these data indicate that variants in this highly conserved domain may have pathological consequences, which might contribute to human disease.

Disease-associated SNVs of the TPNO-1 binding domain of hnRNP A1-M9 result in mis-localization of hnRNP A1 into cytoplasmic stress granules and cellular apoptosis

A total of nine novel SNVs that resulted in an amino acid substitution were discovered in MS patients within the 'core' TPNO-1 binding domain of hnRNP A1-M9. hnRNP A1 has a number of functions, including the transport of nascent mRNA from the nucleus to the cytoplasm. hnRNP A1 shuttles between the nucleus and cytoplasm and binds TPNO-1, which is required for its nuclear import[16,26,27]. At equilibrium, hnRNP A1 is predominantly found in the nucleus[37,38]. Considering the disease-associated SNVs are contained within a highly conserved region of hnRNP A1 that plays a critical role in cellular function, we hypothesized that altered hnRNP A1 would change hnRNP A1 localization, TPNO-1 binding and induce cellular damage. To test this hypothesis, we performed a number of experiments. First, we manufactured three different hnRNP A1 mutations (by site-directed mutagenesis) contained within its TPNO-1 binding domain (F273L, M276L and F281L), transfected each mutant into SK-N-SH cells and examined the cells for hnRNP A1 localization relative to transfection of Wild Type (WT) hnRNP A1. As shown in Figure 2A (upper panel), WT hnRNP A1 almost completely localized to the nucleus of SK-N-SH cells. In contrast, mutant forms of hnRNP A1 localized to the cytoplasm of cells (Figure 2A, lower panel). Localization within the cytoplasm was not diffuse, but granular, suggestive of stress granule (SG) formation (Figure 2A, lower panel, arrows). There was also localization within cellular processes (Figure 2A, lower panel, arrowhead). To confirm that mutant hnRNP A1 was present in SGs, we double-labeled SK-N-SH cells that contained the transfected mutant hnRNP A1 with anti-TDP-43 antibodies. As shown in Figure 2B, like hnRNP A1, TDP-43 was localized to nuclei (without transfection). In addition, WT hnRNP A1 and TDP-43 co-localized within the nuclei of SK-N-SH cells. In contrast, mutant hnRNP A1 (F273L, M276L and F281L) co-localized with TDP-43 within the cytoplasm of cells (Figure 2B). Considering recent data indicating binding between hnRNP A1 and TDP-43; co-localization of mutant hnRNP A1 (p.D262V) to TDP-43 containing SGs; the role of TDP-43 in SG formation, and the localization of hnRNP A1 in SGs in stress activated cells, these experiments confirm that mutant hnRNP A1 is contained within TDP-43 positive SGs[22,39–42]. Because TPNO-1 is required for hnRNP A1 nucleocytoplasmic transport, we hypothesized that mutant

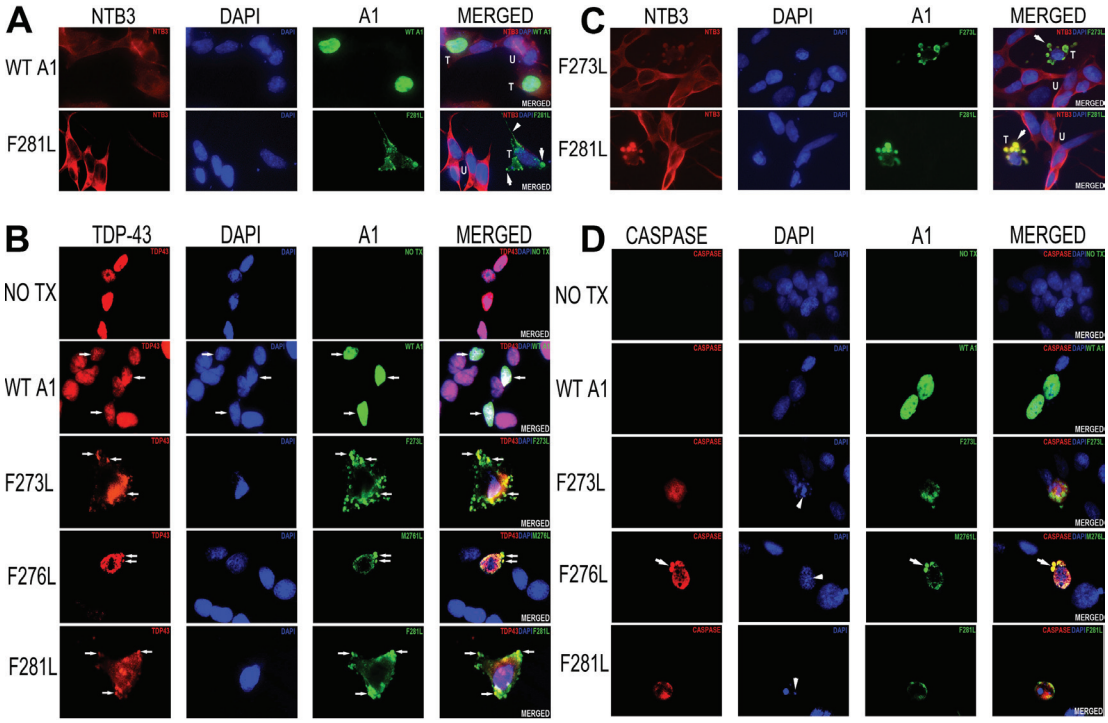

Figure 2. Transfection of WT and mutant forms of hnRNP A1 into SK-N-SH cells. *A*. *Localization of WT and F281L hnRNP A1.* (upper panel) hnRNP A1 localizes to the nuclei of cells transfected (T) with WT hnRNP A1. Untransfected (U) cells are also present in the same field. (Lower panel) In contrast, mutant hnRNP A1 (F281L) mis-localizes to the cytoplasm including cellular processes (arrowhead) in a granular pattern, consistent with stress granule (SGs) formation (arrows) (NTB3-Neuron specific beta III tubulin). ***B***. *Co-localization of hnRNP A1 and TDP-43.* TDP-43 localizes to nuclei of neurons ('no tx') (pink or red signal). WT hnRNP A1 co-localizes with TDP-43 in nuclei (arrows). In contrast, mutant forms of hnRNP A1 (F273L, M276L, F281L) predominantly mis-localize to the cytoplasm and co-localize with TDP-43 positive SGs (arrows, yellow). Some TDP-43 remains in the nucleus (pink or red signal). Figures 2C and D: Apoptosis caused by mutant hnRNP A1 in SK-N-SH neurons. **C**. Transfection of mutant hnRNP A1 (F273L, F281) result in apoptotic blebs in transfected cells (T) (arrows), in contrast to untransfected (U) cells in the same field under identical conditions. **D**. Transfection of WT hnRNP A1 localized to the nucleus and showed no evidence of active caspase-3 labeling. In contrast, mutant hnRNP A1 (F273L, M276L, F281L) predominantly localized to the cytoplasm of cells that stained with active caspase-3, many of which with fragmented nuclei (arrowheads), both of which are indicative of apoptosis. There was also co-localization of active caspase-3 and mutant hnRNP A1 (arrow).

hnRNP A1 would alter its binding to TPNO-1. In these experiments, protein lysates purified from SK-N-SH cells were incubated with either WT or mutant GST-tagged-hnRNP A1 bound to Glutathione-Sepharose 4B beads. The resultant eluent was then probed for TPNO-1. As shown in Figure 3A, western blots showed immunoreactivity for TPNO-1 protein with WT-hnRNP A1, indicative of TPNO-1's binding to hnRNP A1. In contrast, there was significantly reduced binding between mutant forms of hnRNP A1 and TPNO-1 (Figure 3A). These experiments show that mutations in the TPNO-1 binding domain of hnRNP A1-M9 alter TPNO-1 binding to hnRNP A1. To confirm protein binding between hnRNP A1 and TDP-43, which were visualized by immunocytochemistry (Figure 2B), we probed the identical eluents with an anti-TDP-43 antibody. Both WT and mutant hnRNP A1 bound TDP-43 (Figure 3B), indicative of their interaction in both the nuclei and cytoplasmic SGs in the cell line. This is consistent with other reports indicative of an interaction between hnRNP A1 and TDP-43[22].

In the *in-vitro* experiments, SG formation in SK-N-SH cells formed within several hours of transfection. When we waited overnight (approximately 24 hours) the cells containing mutant hnRNP A1 developed apoptotic blebs, which contained hnRNP A1 (Figure 2C, arrows). Apoptosis was confirmed by active caspase-3 staining (Figure 2D). As shown in Figure 2D, SK-N-SH cells transfected with mutant hnRNP A1 showed a cytoplasmic hnRNP A1 distribution, stained positive for active caspase-3 and contained fragment nuclei, confirming apoptosis.

In summary, in contrast to WT hnRNP A1, mutant hnRNP A1 showed markedly reduced binding to its co-receptor TPNO-1, co-localized with TDP-43 within cytoplasmic SGs of cells and caused apoptosis, indicative of the potential pathogenic nature of these disease-associated SNVs in MS patients.

Discussion

Recent studies indicate that in addition to cancer, somatic variants can cause neurological disease[23]. In this study, we discovered novel somatic genomic DNA SNVs in MS patients. Nine were contained within the 'core' TPNO-1 binding domain of hnRNP A1-M9 ($AA^{268-289}$). Three additional SNVs (c.793A>G, p.N265D (in two patients); c.787T>C, p.F263L) included amino acids within the PrLD - M9 overlap region ($AA^{263-267}$), which also bind TPNO-1[45].

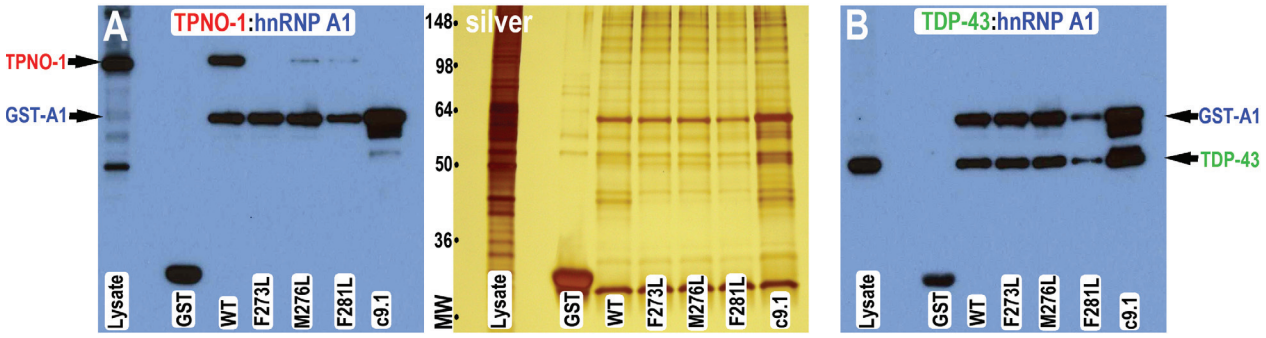

Figure 3. Western blots of hnRNP A1 binding with TPNO-1 and TDP-43. GST-tagged WT or mutant forms of hnRNP A1 were bound to Glutathione Sepharose 4B beads, protein lysates of SK-N-SH cells were applied to columns, and the resulting eluent was probed for either TPNO-1 (**A**) or TDP-43 (**B**). **A**. There was strong binding between WT hnRNP A1 and TPNO-1. In contrast, there was no binding between mutant hnRNP A1 (F273L) and TPNO-1, and markedly reduced binding between mutant hnRNP A1s (M276L, F281L) and TPNO-1. **B**. There was no change in binding between WT and mutant forms of hnRNP A1 and TDP-43.

These variants were in a region of hnRNP A1 that are adjacent to mutations shown to cause ALS (p.D262V, p.N267A). Interestingly, 8 of these 12 SNV's that involved hnRNP A1-M9 binding to TPNO-1 occurred in PPMS patients. In addition, two hnRNP A1 SNVs were contained exclusively within the PrLD (c.755G>A, p.S252N; c.775A>G, p.S259G). There were also six novel SNVs that resulted in an amino acid substitution within the MS IgG epitope of M9 ($AA^{293-304}$), five of which segregated to patients with SPMS. Finally, there were nine SNV's in the C-terminal of hnRNP A1 ($AA^{306-320}$), occurring with similar frequency in HCs and MS patients. The overall somatic SNV rate (based on the number of clones sequenced) for the M9 target sequence was: PPMS - 2.21%, SPMS - 1.69%, RRMS - 0.56%, HC - 0%. If one includes the PrLD (a domain shown to be critical to hnRNP A1 function), the rates increase in PPMS, SPMS and RRMS to 2.84%, 2.25% and 0.84% respectively. None were identical to somatic mutations in the COSMIC database (n = 981,720 samples, n = 1,292,597 unique variants). We utilized a PCR - cloning technique that has been fine-tuned for more than a decade and shows a mutation rate of approximately 0.1% in more than 46,000 clones that were examined[36]. The rates in progressive MS patients exceed this error rate by more than a log. In addition, under identical conditions, there were no mutations in the M9 target sequence or the PrLD domain in the HCs we examined. Thus, these results are unlikely to be due to PCR errors. Importantly, there was little or no overlap with either SNVs or SNPs reported in four different databases.

hnRNP A1 was one of the first RNA binding proteins shown to shuttle into and out of the nucleus[37,38]. Nucleocytoplasmic transport is dependent upon binding between the M9 domain ($AA^{268-305}$) of hnRNP A1 to TPNO-1, in order for this complex to pass through the nuclear pore. M9 acts as both an NES and NLS. M9 is a bipartite PY-NLS whose three-dimensional structure and binding contacts with TPNO-1 are well characterized[26,27,43]. Specifically, M9 contains three binding epitopes (Table 2): a hydrophobic (273-FGPM-276) domain, a basic residue (522R) and a C-terminal RX_{2-5} PY motif, each connected by 'linker' residues[26,27,43]. Each epitope, as well as individual amino acids within an epitope, conveys varying degrees of binding activity to TPNO-1[26,43,44]. For example, mutant P275A dramatically inhibits nucleocytoplasmic transport and substitution of 273-FGPM-276 with 273-AAAA-276, completely abolishes TPNO-1 binding and nucleocytoplasmic transport[37,43,44]. Our data closely align with these findings. For instance, genomic SNVs in MS patients occurred at F273L, F275S, and M276L - all contained within epitope 1. Experimentally, we showed that transfection of F273L and M276L mutants caused mis-localization of hnRNP A1, SG formation (co-localizing with TDP-43), cellular apoptosis and diminution of TPNO-1 binding. Mutant F281L caused similar results. Interestingly, F273, F281 and R284 all have two or more side chains that bind TPNO-1, thus are critical contact points between M9 and TPNO-1[26]. MS patients also had an SNV at R284G. Although we did not test this variant, a parallel substitution in the RNA binding protein fused in sarcoma (FUS) (R522G) (Table 2) caused a five-fold decrease in TPNO-1 binding and cytoplasmic mis-localization of FUS[27]. Like hnRNP A1, FUS is an hnRNP, which at equilibrium localizes to the nucleus and contains a bipartite PY-NLS that binds TPNO-1[27]. Interestingly, mutations in FUS have been shown to cause ALS and frontotemporal lobe dementia[17].

Importantly, none of the SNVs contained within hnRNP A1 - M9 or the PrLD has been reported previously. For decades, the only certain genetic risk factor for MS was with MHC Class II HLA-DRB1[8]. Genome Wide Association Studies (GWAS) have uncovered novel genetic associations with MS[1,2,45] including with the interleukin-2 receptor-α and interleukin-7 receptor genes[45]. Subsequent studies using several thousand MS cases and controls, which analyzed hundreds of thousands of autosomal SNPs, confirmed the association of MS with major MHC Class II HLA-DRB1 (DRB1 *15:01, *15:03, *13:03) and the protective effect MHC Class I HLA-A*02[1,8]. Additional studies showed a total of 48 new and 49 known non-MHC SNPs associated with MS[2]. Interestingly, the functions of the vast majority of the SNP's were related to $CD4^+$ T-lymphocyte and immune regulation[1,8]. This is important, considering the role that T-lymphocytes and the immune response play in the pathogenesis of MS. A few were potentially associated with neurodegeneration[46]. Further, >95% of the SNPs were intronic or intergenic, with only a few SNPs involving exons, in contrast to the somatic SNVs discovered here[1,2]. In addition to GWAS, whole exome sequencing (WES) is being used to examine differential gene expression in MS patients. In contrast to GWAS, which detects known SNPs and utilizes statistical analyses designed to reveal common variants, WES is designed to discover novel, rare pathologic variants[8]. One

Table 2. Binding epitopes of the PY-NLS's of hnRNP A1 and fused in sarcoma (FUS). Aligned PY-NLS sequences of hnRNP A1-M9 and FUS-NLS are shown. The three binding epitopes of the PY-NLS are shaded yellow (epitope 1 - hydrophobic), blue (epitope 2 - basic) and red (epitope 3 - C-terminal)[27,43]. SNVs resulting in an amino acid substitution (hnRNP A1) and missense mutations (FUS) are shown by gray shading of the amino acid sequence number. Amino acid SNVs associated with multiple sclerosis (MS) in hnRNP A1 are shown above the primary sequence and mutations associated with amyotrophic lateral sclerosis (ALS) in FUS are shown below the primary sequence. Two SNVs (*) in hnRNP A1-M9 align with mutations in the FUS NLS: hnRNP A1-M9 AA^{275} with FUS NLS AA^{510} and hnRNP A1-M9 AA^{284} with FUS NLS AA^{522}. Stop codons, frame shift mutations, insertions and deletions not included. Notes: [1]R514G/S, [2]R514S, G515C, [3]R514S, E516V[17].

														*											*					
hnRNP A1 AA sequence #	262	263	264	265	266	267	268	269	270	271	272	273	274	275	276	277	278	279	280	281	282	283	(-)	(-)	(-)	284	285	286	287	288	289
hnRNP A1 AA SNV (MS)		L		D								L		S	L	N			S	L						G	G				
hnRNP A1 AA sequence	D	F	G	N	Y	N	N	Q	S	S	N	F	G	P	M	K	G	G	N	F	G	G	(-)	(-)	(-)	R	S	S	G	P	Y
FUS AA sequence											G	P	G	K	M	D	S	R	G	E	H	R	Q	D	R	R	(-)	E	R	P	Y
FUS AA mutation (ALS)											D			E/R			P	G/S[1,2,3]	C[2]	V[3]	P/Q	G/K			G/H/L	G			S/T/W	L	
FUS AA sequence #											507	508	509	510	511	512	513	514	515	516	517	518	519	520	521	522	(-)	523	524	525	526

of the genes identified by GWAS was CYP27B1, which encodes an enzyme of the same name that converts 25-hydroxyvitamin D to 1,25 hydroxyvitamin D, the biologically active form of vitamin D[1,8]. A single individual from 43 MS families was found to have a rare p.R389H genetic variant in CYP27B1, which resulted in complete loss of enzyme activity[8,47,48]. However, unaffected relatives of the individual also carried the identical variant, which has a high frequency in the general population[48].

Thus, it is clear that an individual's genetic background makes an important contribution to the pathogenesis of MS. This supports the tripartite hypothesis that an environmental trigger in a genetically susceptible individual causes an autoimmune response to CNS antigens that result in the pathology observed in the brain and spinal cord of MS patients. Potential environmental triggers include viral infection, low vitamin D levels and sun exposure[49–51]. During the relapsing phase of MS, Th1 and Th17 $CD4^+$ T-lymphocyte responses appear to predominate and correlate with focal MS plaque formation in the CNS[5,52,53]. As MS evolves into a secondary progressive phase, CNS damage becomes more diffuse[5,6,53]. Immune cells also become more diffuse and IgG containing plasma cells, B-lymphocytes and macrophage/microglia response predominate[7,53]. Many of these latter features are also characteristic of primary progressive MS[7]. MS is also characterized by increased oxidative stress (in PBMC and brain), which can cause DNA damage and somatic mutations[54–56]. Further, T-lymphocytes isolated from MS patients that contain somatic mutations have been shown to react to myelin peptides[57]. Specifically, T-cell clones mutant for the hypoxanthine guanine phosphoribosyltransferase (HPRT) gene were found only in MS patients and not in HCs[57,58]. The MS patients studied had 'chronic progressive MS', clinically similar to the PPMS patients in this study[57]. The T-cell clones recognized and proliferated to myelin basic protein peptides[57]. These data suggest that T-cells that undergo clonal expansion are more prone to somatic mutations and that clones containing somatic mutations contribute to the pathogenesis of MS[57,58]. How might variants in hnRNP A1 contribute to the pathogenesis of MS? Although direct evidence is not yet available and requires further study, the molecular consequences of abnormal forms of hnRNP A1 on cellular function may have profound effects on the immune system. For example, as it relates to the environment, hnRNP A1 regulates the synthesis of several viruses including human immunodeficiency virus, HTLV-1, and human rhinovirus[59–61]. Immunologically, hnRNP A1's nucleocytoplasmic shuttling and RNA binding specificity is required for myelopoiesis and modulation of immune-mediated programmed cell death[62–64]. Further, apoptotic blebs (which we showed in this study to contain hnRNP A1) have profound effects on the immune response, as they are believed to initiate and perpetuate autoimmune diseases such as systemic lupus erythematosus[65,66].

In addition, genetic mutations in hnRNP A1 alter neuronal function. For example, an inherited mutation in the PrLD of hnRNP A1 resulted in its mis-localization to TDP-43 positive cytoplasmic SGs and thus is thought to cause familial ALS[22]. A second mutation in the PrLD was also discovered in a sporadic case of ALS[22]. Interestingly, in addition to inherited and germ line mutations (including SNPs), recent data indicate that acquired mutations cause neurological disease[23]. For example, both familial (inherited) and acquired ('*de novo*', somatic variants) of doublecortin cause subcortical band heterotopia (SBH), a neuronal migration syndrome that results in epilepsy and intellectual disability[67]. In contrast to inherited mutations, somatic mutations may only be present in specific cell lineages[23]. In SBH, somatic mutations are only found in the DNA of neurons and lymphocytes, and not in other tissues (a 'mosaic')[23,67]. To date, we have only discovered somatic mutations in PBMCs of MS patients. Future studies will address their presence in CNS tissues of MS patients. If present in the CNS, abnormal forms of hnRNP A1, which have profound effects on neurons, might also damage astrocytes and oligodendroglia, and could contribute to neurodegeneration present in MS, particularly in PPMS where the majority of the SNVs were located.

In summary, we discovered novel SNVs in MS patients. The SNVs involve the M9 nucleocytoplasmic binding domain of hnRNP A1, which when transfected into a cell line, resulted mis-localization of hnRNP A1 to cytoplasmic stress granules and cellular apoptosis. Future studies are required to replicate this data, expand it to include a broader spectrum of genes, a greater number of MS patients and patients with other chronic inflammatory diseases such as rheumatoid arthritis. In addition, we also plan to perform functional studies of the SNVs in immune and nervous system cells of MS patients.

Data availability

Single nucleotide variants (SNVs) (somatic) in MS patients submitted to dbSNP[68]

#	HGVS NAME	LOCAL ID	ss
1	NM_002136.2:c.755G>A	NP_002127.1:c.755G>A	ss1056389899
2	NM_002136.2:c.775A>G	NP_002127.1:c.775A>G	ss1056389900
3	NM_002136.2:c.787T>C	NP_002127.1:c.787T>C	ss1056389901
4	NM_002136.2:c.793A>G	NP_002127.1:c.793A>G	ss1056389902
5	NM_002136.2:c.826A>C	NP_002127.1:c.826A>C	ss1056389903
6	NM_002136.2:c.839A>G	NP_002127.1:c.839A>G	ss1056389904
7	NM_002136.2:c.937A>G	NP_002127.1:c.937A>G	ss1056389905
8	NM_002136.2:c.940T>C	NP_002127.1:c.940T>C	ss1056389906
9	NM_002136.2:c.955A>G	NP_002127.1:c.955A>G	ss1056389907
10	NM_002136.2:c.817T>G	NP_002127.1:c.817T>G	ss1056389908
11	NM_002136.2:c.817T>C	NP_002127.1:c.817T>C	ss1056389909
12	NM_002136.2:c.823C>T	NP_002127.1:c.823C>T	ss1056389910
13	NM_002136.2:c.831G>T	NP_002127.1:c.831G>T	ss1056389911
14	NM_002136.2:c.841T>C	NP_002127.1:c.841T>C	ss1056389912
15	NM_002136.2:c.850A>G	NP_002127.1:c.850A>G	ss1056389913
16	NM_002136.2:c.853A>G	NP_002127.1:c.853A>G	ss1056389914
17	NM_002136.2:c.884A>G	NP_002127.1:c.884A>G	ss1056389915
18	NM_002136.2:c.886T>C	NP_002127.1:c.886T>C	ss1056389916
19	NM_002136.2:c.896C>T	NP_002127.1:c.896C>T	ss1056389917
20	NM_002136.2:c.898C>T	NP_002127.1:c.898C>T	ss1056389918
21	NM_002136.2:c.902A>G	NP_002127.1:c.902A>G	ss1056389919
22	NM_002136.2:c.901A>G	NP_002127.1:c.901A>G	ss1056389920
23	NM_002136.2:c.922T>G	NP_002127.1:c.922T>G	ss1056389921
24	NM_002136.2:c.941A>G	NP_002127.1:c.941A>G	ss1056389922
25	NM_002136.2:c.950G>A	NP_002127.1:c.950G>A	ss1056389923
26	NM_002136.2:c.958T>C	NP_002127.1:c.958T>C	ss1056389924

Author contributions

SL and MCL conceived the study. SL and MCL designed the experiments. SL carried out the bench research. MCL prepared the first draft of the manuscript. SL and MCL contributed to the experimental design and preparation of the manuscript. All authors were involved in the revision of the draft manuscript and have agreed to the final content.

Competing interests

Drs. Michael Levin and Sangmin Lee have a United States patent pending titled "Biomarker for neurodegeneration in neurological disease".

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Supplementary materials

Supplement 1: Patient demographics (xlsx)

Supplement 2: Single nucleotide variants in the target sequence of hnRNP A1 (xlsx)

Supplement 3: Number of clones containing single nucleotide variants (xlsx)

References

1. Sawcer S, Hellenthal G, Pirinen M, *et al.*: **Genetic risk and a primary role for cell-mediated immune mechanisms in multiple sclerosis.** *Nature.* 2011; **476**(7359): 214–219.
PubMed Abstract | Publisher Full Text | Free Full Text
2. Beecham AH, Patsopoulos NA, Xifara DK, *et al.*: **Analysis of immune-related loci identifies 48 new susceptibility variants for multiple sclerosis.** *Nat Genet.* 2013; **45**(11): 1353–1360.
PubMed Abstract | Publisher Full Text | Free Full Text
3. Levin MC, Lee S, Gardner L, *et al.*: **Pathogenic mechanisms of neurodegeneration based on the phenotypic expression of progressive forms of immune-mediated neurologic disease.** *Degenerat Neurolog Neuromusc Dis.* 2012; **2**: 175–187.
Publisher Full Text
4. Levin MC, Douglas J, Myers L, *et al.*: **Neurodegeneration in multiple sclerosis involves multiple pathogenic mechanisms.** *Degenerative Neurolog Neuromusc Dis.* 2014; **4**: 49–63.
Publisher Full Text
5. Lassmann H, van Horssen J: **The molecular basis of neurodegeneration in multiple sclerosis.** *FEBS Lett.* 2011; **585**(23): 3715–23.
PubMed Abstract | Publisher Full Text
6. Dutta R, Trapp BD: **Mechanisms of neuronal dysfunction and degeneration in multiple sclerosis.** *Prog Neurobiol.* 2011; **93**(1): 1–12.
PubMed Abstract | Publisher Full Text | Free Full Text
7. Antel J, Antel S, Caramanos Z, *et al.*: **Primary progressive multiple sclerosis: part of the MS disease spectrum or separate disease entity?** *Acta Neuropathol.* 2012; **123**(5): 627–38.
PubMed Abstract | Publisher Full Text
8. Gourraud PA, Harbo HF, Hauser SL, *et al.*: **The genetics of multiple sclerosis: an up-to-date review.** *Immunol Rev.* 2012; **248**(1): 87–103.
PubMed Abstract | Publisher Full Text
9. Levin MC, Lee S, Gardner LA, *et al.*: **Autoantibodies to Non-myelin Antigens as Contributors to the Pathogenesis of Multiple Sclerosis.** *J Clin Cell Immunol.* 2013; **4**.
PubMed Abstract | Publisher Full Text | Free Full Text
10. Obermeier B, Lovato L, Mentele R, *et al.*: **Related B cell clones that populate the CSF and CNS of patients with multiple sclerosis produce CSF immunoglobulin.** *J Neuroimmunol.* 2011; **233**(1–2): 245–8.
PubMed Abstract | Publisher Full Text | Free Full Text
11. Gabibov AG, Belogurov AA Jr, Lomakin YA, *et al.*: **Combinatorial antibody library from multiple sclerosis patients reveals antibodies that cross-react with myelin basic protein and EBV antigen.** *FASEB J.* 2011; **25**(12): 4211–21.
PubMed Abstract | Publisher Full Text
12. Derfuss T, Linington C, Hohlfeld R, *et al.*: **Axo-glial antigens as targets in multiple sclerosis: implications for axonal and grey matter injury.** *J Mol Med (Berl).* 2010; **88**(8): 753–61.
PubMed Abstract | Publisher Full Text
13. Mathey EK, Derfuss T, Storch MK, *et al.*: **Neurofascin as a novel target for autoantibody-mediated axonal injury.** *J Exp Med.* 2007; **204**(10): 2363–72.
PubMed Abstract | Publisher Full Text | Free Full Text
14. Silber E, Semra YK, Gregson NA, *et al.*: **Patients with progressive multiple sclerosis have elevated antibodies to neurofilament subunit.** *Neurology.* 2002; **58**(9): 1372–81.
PubMed Abstract | Publisher Full Text
15. Huizinga R, Linington C, Amor S: **Resistance is futile: antineuronal autoimmunity in multiple sclerosis.** *Trends Immunol.* 2008; **29**(2): 54–60.
PubMed Abstract | Publisher Full Text
16. Lee S, Xu L, Shin Y, *et al.*: **A potential link between autoimmunity and neurodegeneration in immune-mediated neurological disease.** *J Neuroimmunol.* 2011; **235**(1–2): 56–69.
PubMed Abstract | Publisher Full Text
17. Bentmann E, Haass C, Dormann D: **Stress granules in neurodegeneration--lessons learnt from TAR DNA binding protein of 43 kDa and fused in sarcoma.** *FEBS J.* 2013; **280**(18): 4348–70.
PubMed Abstract | Publisher Full Text
18. Ramaswami M, Taylor JP, Parker R: **Altered ribostasis: RNA-protein granules in degenerative disorders.** *Cell.* 2013; **154**(4): 727–36.
PubMed Abstract | Publisher Full Text | Free Full Text
19. Kiebler MA, Bassell GJ: **Neuronal RNA granules: movers and makers.** *Neuron.* 2006; **51**(6): 685–90.
PubMed Abstract | Publisher Full Text
20. Chen-Plotkin AS, Lee VM, Trojanowski JQ: **TAR DNA-binding protein 43 in neurodegenerative disease.** *Nat Rev Neurol.* 2010; **6**(4): 211–20.
PubMed Abstract | Publisher Full Text | Free Full Text
21. Li YR, King OD, Shorter J, *et al.*: **Stress granules as crucibles of ALS pathogenesis.** *J Cell Biol.* 2013; **201**(3): 361–72.
PubMed Abstract | Publisher Full Text | Free Full Text
22. Kim HJ, Kim NC, Wang YD, *et al.*: **Mutations in prion-like domains in hnRNPA2B1 and hnRNPA1 cause multisystem proteinopathy and ALS.** *Nature.* 2013; **495**(7442): 467–73.
PubMed Abstract | Publisher Full Text | Free Full Text
23. Poduri A, Evrony GD, Cai X, *et al.*: **Somatic mutation, genomic variation, and neurological disease.** *Science.* 2013; **341**(6141): 1237758.
PubMed Abstract | Publisher Full Text | Free Full Text
24. Dreyfuss G, Kim VN, Kataoka N: **Messenger-RNA-binding proteins and the messages they carry.** *Nat Rev Mol Cell Biol.* 2002; **3**(3): 195–205.
PubMed Abstract | Publisher Full Text

25. Bekenstein U, Soreq H: **Heterogeneous nuclear ribonucleoprotein A1 in health and neurodegenerative disease: from structural insights to post-transcriptional regulatory roles.** *Mol Cell Neurosci.* 2013; **56**: 436–46.
PubMed Abstract | Publisher Full Text

26. Lee BJ, Cansizoglu AE, Suel KE, *et al.*: **Rules for nuclear localization sequence recognition by karyopherin beta 2.** *Cell.* 2006; **126**(3): 543–58.
PubMed Abstract | Publisher Full Text | Free Full Text

27. Zhang ZC, Chook YM: **Structural and energetic basis of ALS-causing mutations in the atypical proline-tyrosine nuclear localization signal of the Fused in Sarcoma protein (FUS).** *Proc Natl Acad Sci U S A.* 2012; **109**(30): 12017–21.
PubMed Abstract | Publisher Full Text | Free Full Text

28. Levin M, Lehky T, Flerlage N, *et al.*: **Immunopathogenesis of HTLV-1 associated neurologic disease based on a spinal cord biopsy from a patient with HTLV-1 associated myelopathy/tropical spastic paraparesis (HAM/TSP).** *New Eng J Med.* 1997; **336**: 839–845.

29. Lee SM, Morocos Y, Jang H, *et al.*: **HTLV-1 induced molecular mimicry in neurologic disease**, in Molecular Mimicry: Infection Inducing Autoimmune Disease, M. Oldstone, Editor. 2005, Springer: New York.
PubMed Abstract | Publisher Full Text

30. Lee S, Levin MC: **Molecular mimicry in neurological disease: what is the evidence?** *Cell Mol Life Sci.* 2008; **65**(7–8): 1161–75.
PubMed Abstract | Publisher Full Text

31. Levin MC, Lee SM, Kalume F, *et al.*: **Autoimmunity due to molecular mimicry as a cause of neurological disease.** *Nat Med.* 2002; **8**(5): 509–13.
PubMed Abstract | Publisher Full Text | Free Full Text

32. Lee SM, Dunnavant FD, Jang H, *et al.*: **Autoantibodies that recognize functional domains of hnRNPA1 implicate molecular mimicry in the pathogenesis of neurological disease.** *Neurosci Lett.* 2006; **401**(1–2): 188–93.
PubMed Abstract | Publisher Full Text | Free Full Text

33. Kalume F, Lee SM, Morcos Y, *et al.*: **Molecular mimicry: cross-reactive antibodies from patients with immune-mediated neurologic disease inhibit neuronal firing.** *J Neurosci Res.* 2004; **77**(1): 82–9.
PubMed Abstract | Publisher Full Text

34. Douglas JN, Gardner LA, Levin MC: **Antibodies to an intracellular antigen penetrate neuronal cells and cause deleterious effects.** *J Clin Cell Immunol.* 2013; **4**(1): 134.
Publisher Full Text

35. Polman CH, Reingold SC, Banwell B, *et al.*: **Diagnostic criteria for multiple sclerosis: 2010 revisions to the McDonald criteria.** *Ann Neurol.* 2011; **69**(2): 292–302.
PubMed Abstract | Publisher Full Text | Free Full Text

36. Kitabayashi M, Nishiya Y, Esaka M, *et al.*: **Gene cloning and polymerase chain reaction with proliferating cell nuclear antigen from Thermococcus kodakaraensis KOD1.** *Biosci Biotechnol Biochem.* 2002; **66**(10); 2194–200.
PubMed Abstract | Publisher Full Text

37. Michael WM, Choi M, Dreyfuss G: **A nuclear export signal in hnRNP A1: a signal-mediated, temperature-dependent nuclear protein export pathway.** *Cell.* 1995; **83**(3): 415–22.
PubMed Abstract | Publisher Full Text

38. Siomi H, Dreyfuss G: **A nuclear localization domain in the hnRNP A1 protein.** *J Cell Biol.* 1995; **129**(3): 551–60.
PubMed Abstract | Publisher Full Text | Free Full Text

39. Guil S, Long JC, Caceres JF: **hnRNP A1 relocalization to the stress granules reflects a role in the stress response.** *Mol Cell Biol.* 2006; **26**(15): 5744–58.
PubMed Abstract | Publisher Full Text | Free Full Text

40. Liu-Yesucevitz L, Bilgutay A, Zhang YJ, *et al.*: **Tar DNA binding protein-43 (TDP-43) associates with stress granules: analysis of cultured cells and pathological brain tissue.** *PLoS One.* 2010; **5**(10): e13250.
PubMed Abstract | Publisher Full Text | Free Full Text

41. Lagier-Tourenne C, Polymenidou M, Cleveland DW: **TDP-43 and FUS/TLS: emerging roles in RNA processing and neurodegeneration.** *Hum Mol Genet.* 2010; **19**(R1): R46–64.
PubMed Abstract | Publisher Full Text | Free Full Text

42. Ling SC, Polymenidou M, Cleveland DW: **Converging mechanisms in ALS and FTD: disrupted RNA and protein homeostasis.** *Neuron.* 2013; **79**(3): 416–38.
PubMed Abstract | Publisher Full Text

43. Xu D, Farmer A, Chook YM: **Recognition of nuclear targeting signals by Karyopherin-β proteins.** *Curr Opin Struct Biol.* 2010; **20**(6): 782–90.
PubMed Abstract | Publisher Full Text | Free Full Text

44. Bogerd HP, Benson RE, Truant R, *et al.*: **Definition of a consensus transportin-specific nucleocytoplasmic transport signal.** *J Biol Chem.* 1999; **274**(14): 9771–7.
PubMed Abstract | Publisher Full Text

45. Hafler DA, Compston A, Sawcer S, *et al.*: **Risk alleles for multiple sclerosis identified by a genomewide study.** *N Engl J Med.* 2007; **357**(9): 851–62.
PubMed Abstract | Publisher Full Text

46. Bush WS, McCauley JL, DeJager PL, *et al.*: **A knowledge-driven interaction analysis reveals potential neurodegenerative mechanism of multiple sclerosis susceptibility.** *Genes Immun.* 2011; **12**(5): 335–40.
PubMed Abstract | Publisher Full Text | Free Full Text

47. Ramagopalan SV, Dyment DA, Cader MZ, *et al.*: **Rare variants in the CYP27B1 gene are associated with multiple sclerosis.** *Ann Neurol.* 2011; **70**(6): 881–6.
PubMed Abstract | Publisher Full Text

48. Oksenberg JR, Hauser SL: **Decoding multiple sclerosis.** *Ann Neurol.* 2011; **70**(6): A5–7.
PubMed Abstract | Publisher Full Text

49. Munger KL, Levin LI, Hollis BW, *et al.*: **Serum 25-hydroxyvitamin D levels and risk of multiple sclerosis.** *JAMA.* 2006; **296**(23): 2832–8.
PubMed Abstract | Publisher Full Text

50. Ascherio A, Munger KL: **Environmental risk factors for multiple sclerosis. Part I: the role of infection.** *Ann Neurol.* 2007; **61**(4): 288–99.
PubMed Abstract | Publisher Full Text

51. Ascherio A, Munger KL, Simon KC: **Vitamin D and multiple sclerosis.** *Lancet Neurol.* 2010; **9**(6): 599–612.
PubMed Abstract | Publisher Full Text

52. Kleinewietfeld M, Manzel A, Titze J, *et al.*: **Sodium chloride drives autoimmune disease by the induction of pathogenic TH17 cells.** *Nature.* 2013; **496**(7446): 518–22.
PubMed Abstract | Publisher Full Text | Free Full Text

53. Lassmann H, van Horssen J, Mahad D: **Progressive multiple sclerosis: pathology and pathogenesis.** *Nat Rev Neurol.* 2012; **8**(11): 647–56.
PubMed Abstract | Publisher Full Text

54. Haider LC, Simeonidou G, Steinberger S, *et al.*: **Multiple sclerosis deep grey matter: the relation between demyelination, neurodegeneration, inflammation and iron.** *J Neurol Neurosurg Psychiatry.* 2014.
PubMed Abstract | Publisher Full Text

55. Sadowska-Bartosz I, Adamczyk-Sowa M, Gajewska A, *et al.*: **Oxidative modification of blood serum proteins in multiple sclerosis after interferon or mitoxantrone treatment.** *J Neuroimmunol.* 2014; **266**(1–2): 67–74.
PubMed Abstract | Publisher Full Text

56. Dizdaroglu M: **Oxidatively induced DNA damage: mechanisms, repair and disease.** *Cancer Lett.* 2012; **327**(1–2): 26–47.
PubMed Abstract | Publisher Full Text

57. Lodge PA, Allegretta M, Steinman L, *et al.*: **Myelin basic protein peptide specificity and T-cell receptor gene usage of HPRT mutant T-cell clones in patients with multiple sclerosis.** *Ann Neurol.* 1994; **36**(5): 734–40.
PubMed Abstract | Publisher Full Text

58. Allegretta M, Nicklas JA, Sriram S, *et al.*: **T cells responsive to myelin basic protein in patients with multiple sclerosis.** *Science.* 1990; **247**(4943): 718–21.
PubMed Abstract | Publisher Full Text

59. Najera I, Krieg M, Karn J: **Synergistic stimulation of HIV-1 rev-dependent export of unspliced mRNA to the cytoplasm by hnRNP A1.** *J Mol Biol.* 1999; **285**(5): 1951–64.
PubMed Abstract | Publisher Full Text

60. Kress E, Baydoun HH, Bex F, *et al.*: **Critical role of hnRNP A1 in HTLV-1 replication in human transformed T lymphocytes.** *Retrovirology.* 2005; **2**: 8.
PubMed Abstract | Publisher Full Text | Free Full Text

61. Cammas A, Pileur F, Bonnal S, *et al.*: **Cytoplasmic relocalization of heterogeneous nuclear ribonucleoprotein A1 controls translation initiation of specific mRNAs.** *Mol Biol Cell.* 2007; **18**(12): 5048–59.
PubMed Abstract | Publisher Full Text | Free Full Text

62. Iervolino A, Santilli G, Trotta R, *et al.*: **hnRNP A1 nucleocytoplasmic shuttling activity is required for normal myelopoiesis and BCR/ABL leukemogenesis.** *Mol Cell Biol.* 2002; **22**(7): 2255–66.
PubMed Abstract | Publisher Full Text | Free Full Text

63. Hamilton BJ, Burns CM, Nichols RC, *et al.*: **Modulation of AUUUA response element binding by heterogeneous nuclear ribonucleoprotein A1 in human T lymphocytes. The roles of cytoplasmic location, transcription, and phosphorylation.** *J Biol Chem.* 1997; **272**(45): 28732–41.
PubMed Abstract | Publisher Full Text

64. Rajani DK, Walch M, Martinvalet D, *et al.*: **Alterations in RNA processing during immune-mediated programmed cell death.** *Proc Natl Acad Sci U S A.* 2012; **109**(22): 8688–93.
PubMed Abstract | Publisher Full Text | Free Full Text

65. Radic MZ, Shah K, Zhang W, *et al.*: **Heterogeneous nuclear ribonucleoprotein P2 is an autoantibody target in mice deficient for Mer Axl, and Tyro3 receptor tyrosine kinases.** *J Immunol.* 2006; **176**(1): 68–74.
PubMed Abstract | Publisher Full Text | Free Full Text

66. Wickman G, Julian L, Olson MF: **How apoptotic cells aid in the removal of their own cold dead bodies.** *Cell Death Differ.* 2012; **19**(5): 735–42.
PubMed Abstract | Publisher Full Text | Free Full Text

67. Bahi-Buisson N, Souville I, Fourniol FJ, *et al.*: **New insights into genotype-phenotype correlations for the doublecortin-related lissencephaly spectrum.** *Brain.* 2013; **136**(Pt 1): 223–44.
PubMed Abstract | Publisher Full Text | Free Full Text

68. **Database of Single Nucleotide Polymorphisms (dbSNP)**. Bethesda (MD): National Center for Biotechnology Information, National Library of Medicine. dbSNP accession:[ss1056389899 - ss1056389924], (dbSNP Build ID: [141]).
Reference Source

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Referee Responses for Version 1

Elliot M. Frohman
Department of Neurology, University of Texas Southwestern Medical Center at Dallas, Dallas, USA

Approved: 10 September 2014

Referee Report: 10 September 2014
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My distinguished colleagues from the University of Tennessee in Memphis have performed an innovative set of complex studies seeking to elucidate somatic mutation variants in patients with MS; across the various clinical subtypes, and compared to normal subjects. Quite surprising to this reviewer, the authors identified a TPNO-1 binding domain change of hnRNPA1-M9; an RNA binding protein that is among the first to be shuttled into and out of the nucleus. Perhaps even more conspicuous is that these somatic modifications are directly adjacent to well characterized mutations that are associated with the development of ALS (i.e. accelerated motor neuron disease of the upper and lower motor neuron compartments).

My colleagues further show that 8/12 SNVs are shown to occur in primary progressive MS (a slowly and insidiously progressive loss of the upper motor neuron compartment of the CNS. They further elucidated six novel SNVs; with substitutions within the MS IgG epitope of M9 (AA 293-304).

The authors proceed to provide a SNV rate stratification across normal subjects vs clinical subtypes of MS. Here they found the following corresponding rates:

PPMS- 2.21%
SPMS- 1.69%
RRMS- 0.56%
Normal human subjects = 0%

Much has been achieved with the application of genome wide association studies (GWAS), such as the identification of a linkage between the IL-2 and IL-7 receptor genes; immune regulatory elements that dovetail nicely with the higher risk of MS developing in subjects who are also positive for the MHC Class II HLADR21501B1 (2 copies > 1 copy with respect to relative risk). Alternately, MHC Class I HLA-A*02 confers a protective influence. Indeed, it is intriguing that most of the SNPs are associated with CD4+T cell regulation.

Contemporary screening techniques have further evolved with even greater refinement in genetic element identification conferring risk of MS by virtue of the utilization of the powerful technique of whole exome sequencing (WES), that provides important perspective on differential gene expression mechanisms.

Finally, the GWAS discovery associated with MS of the CYP27B1 gene, has particular relevance to an

epigenetic disorder such as MS, given that this gene encodes for the critical enzyme that serves to transform the 25-OH-D form to the 1,25-OH-D active form of vitamin D.

With respect to the pathophysiologic relevance of the hnRNPA1 variants, these appear to alter neuronal function, and ultimately culminate in neurodegeneration, in conjunction with serving a role in the important regulation of viral synthesis (another highly conspicuous epigenetic factor with special relevance to MS and related disorders). As the authors rightly point out, to date the somatic mutations in question and under investigation have only been elucidated from PBMCs of MS patients.

This work, if confirmed, may have special and critical relevance to the highly enigmatic phenomenology of what determine the transition from a relapsing to a progressive form of MS (or a progressive form from the inception without an antecedent relapsing and adaptive immune phase of the disorder), and such studies might shed light on the pathobiological underpinnings of axonal and neuronal degeneration; the histopathological signatures of what we currently believe to constitute the signature of irreversible (at least so far) neurologic disability with corresponding loss of functional capabilities.

This work, contributes to our deeper understanding of the potential mechanisms of disease that figure prominently in influencing progression and neurodegeneration for MS in particular, but may also shed light on those mechanisms that underly neurodegenerative disorders in general.

The authors are to be congratulated for this fine contribution to the literature.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Competing Interests: No competing interests were disclosed.

1 Comment

Author Response

Michael Levin, University of Tennessee Health Science Center, Memphis, USA
Posted: 11 Sep 2014

We appreciate Professor Frohman's insightful commentary about our work. We concur that to date, we have only found somatic nucleotide variants (SNVs) in PBMCs of MS patients. Further studies are warranted about the contribution of SNVs to axonal and neuronal degeneration in the CNS of MS patients. This is addressed in the last two paragraphs of the discussion.
Competing Interests: No competing interests.

Michael K. Racke
Department of Neurology and Multiple Sclerosis Center, The Ohio State University, Columbus, OH, USA

Approved: 23 July 2014

Referee Report: 23 July 2014
doi:10.5256/f1000research.4747.r5207

This manuscript by Lee and Levin continues work by Levin on the role of an immune response to hnRNP-A1 in human demyelinating disorders, including multiple sclerosis. Previously, Levin had shown that an antibody response to HTLV-I Tax cross-reacted with hnRNP-A1. Now, they go on to show that there are mutations in hnRNP-A1 that affect the localization of this protein and how it could affect neuronal survival in multiple sclerosis.In general, the experiments appear to be well performed. One might be interested to know whether testing in another cell line such as a oligodendroglial cell line would have similar effects on cell survival as in the neuroblastoma cell line. This could also be highly relevant in MS, as in addition to neuronal loss, there is demyelination and loss of oligodendrocytes.This work attempts to address whether mutations in hnRNP-A1 could contribute to MS pathogenesis. It would be interesting to note whether antibody responses to the protein correlated with mutations (i.e. does the mutation in the protein affect tolerance). Another issue that needs to be addressed is whether the mutations in the hnRNP-A1 gene are due to increased mutational frequency that can be observed in replicating cells, similar to the observation of increased hprt mutations made by Allegretta many years ago in MBP-specific T cells.Overall, this is an interesting study which certainly increases the interest in non-myelin targets in diseases such as MS.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Competing Interests: No competing interests were disclosed.

1 Comment

Author Response

Michael Levin, University of Tennessee Health Science Center, Memphis, USA
Posted: 11 Sep 2014

We appreciate Professor Racke's critique, including that the studies were "well performed." The following concerns expressed by Professor Racke were addressed:

1. **One might be interested to know whether testing in another cell line such as a oligodendroglial cell line would have similar effects on cell survival as in the neuroblastoma cell line. This could also be highly relevant in MS, as in addition to neuronal loss, there is demyelination and loss of oligodendrocytes.**

   We used SK-N-SH neurons as a model system to examine the effect that mutant forms of hnRNP A1 might have on target cell function. Considering that cells other than neurons are clearly involved in the pathogenesis of MS, we plan to add oligodendrocyte cell lines to future studies of mutant hnRNP A1. We addressed this in the sixth paragraph of the discussion.

2. **This work attempts to address whether mutations in hnRNP-A1 could contribute to MS pathogenesis. It would be interesting to note whether antibody responses to the**

protein correlated with mutations (i.e. does the mutation in the protein affect tolerance).

We agree that this would be in important and insightful experiment, one that we think is beyond the scope of the current study. Our previous work showed that MS patients develop antibodies to hnRNP A1 using Western blots. We have not yet developed the technology that would allow us to quantitate the amount of antibodies to hnRNP A1 in individual patients. We will consider this in the future as we examine the potential connection between antibodies to and somatic mutations within hnRNP A1.

3. **Another issue that needs to be addressed is whether the mutations in the hnRNP-A1 gene are due to increased mutational frequency that can be observed in replicating cells, similar to the observation of increased hprt mutations made by Allegretta many years ago in MBP-specific T cells.**

   We appreciate this insight offered by Professor Racke, which is very relevant to our work and have included it in paragraph 4 of the discussion.

Competing Interests: No competing interests.

Hans Lassmann
Department Neuroimmunology, Medical University of Vienna, Wien, A-1090, Austria

Approved with reservations: 03 July 2014

Referee Report: 03 July 2014
doi:10.5256/f1000research.4747.r5210

This is a potentially interesting study, reporting increased presence of novel somatic single nucleotide variants (SNVs) within the RNA binding protein hnRNP A1 in peripheral blood mononuclear cells (PBMCs) in patients with multiple sclerosis (MS) in comparison to controls. The disease associated SNVs resulted in a mis-localization of hnRNP A1 into cytoplasmic stress granules and this mis-localization was associated with cellular apoptosis in cells, transfected with the respective mutants. From a technical point of view the study is well performed. The question, however, remains what the findings mean for the pathogenesis of multiple sclerosis.It is interesting to see that there is apparently a high incidence of induced SNVs in PBMCs of MS patients, which most likely not only affects the gene investigated in this study. This may be due to the fact that MS is a chronic inflammatory disease, leading to proliferation of leukocytes (in particular T-lymphocytes) in the peripheral immune system. In addition increased oxidative stress in PBMCs of MS patients has been reported before. Both conditions may lead to increased somatic mutation in these cell populations. Thus, in a study like this the inclusion of a control population with long-lasting chronic inflammation would be important in addition to the study of normal controls. According to the presented data, the particular SNVs are also likely to result in cell elimination. Whether this changes the immune response in MS patients has not been investigated in this study.Finally the authors discuss that their findings may have implications for the understanding the mechanisms of tissue injury and neurodegeneration in MS. To support this conclusion, evidence has to be provided that similar

somatic mutations are also present in cells of the central nervous system, such as neurons or oligodendrocytes. The basic observation of an increased incidence of somatic mutations in PBMCs in MS presented in this article is a first (but preliminary) observation, which is interesting.However, it is not clear what this means for the disease. The key message from the authors that this is an important finding for MS pathogenesis is not substantiated by the data. I agree with the authors that further experiments (possibly beyond the scope of a new version of this article) are needed. For example:

1. To look for somatic mutations for other genes, to see whether the paper just shows that in PBMCs of MS patients there is an increased rate of somatic mutations in many different genes or whether the mutations in the specific gene are MS specific.

2. To expand the sample of control patients, in particular by including patients with other chronic inflammatory diseases (e.g. patients with chronic rheumatoid arthritis).

3. To search for similar mutations in neurons and glia of the MS brain to establish a link to neurodegeneration in this disease.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Competing Interests: No competing interests were disclosed.

1 Comment

Author Response

Michael Levin, University of Tennessee Health Science Center, Memphis, USA
Posted: 11 Sep 2014

We appreciate Professor Lassmann's comments including that the study was "well performed" and the acknowledgement that "increased incidence of somatic mutations in PBMCs in MS presented in this article is a first…". We agree with his constructive critiques, which we have addressed below.

1. **To look for somatic mutations for other genes, to see whether the paper just shows that in PBMCs of MS patients there is an increased rate of somatic mutations in many different genes or whether the mutations in the specific gene are MS specific.**

   Our ongoing studies are designed to evaluate more genes. Using next-generation sequencing, which is beyond the scope of this paper, we may be able to assess a broader number of genes, potentially including coding exomes of the human genome. In this study, we evaluated three different regions of hnRNP A1: the C-terminal of PrLD, M9 and hnRNP A1's C-terminus. Within this subset of genes, we saw a segregation of SNVs. For example, there were similar numbers of SNVs in hnRNP A1's C-terminus in all groups examined (Table 1, Supplement 2). In contrast, in PPMS SNVs segregated to the TPNO-1 binding domain of M9 and SPMS to the MS IgG epitope of M9. Although more genes are required to assess their role in MS compared to other diseases, in this study, we have already observed a differential expression of SNVs between types of MS and HCs. This is addressed in the

last paragraph of the discussion.

2. **To expand the sample of control patients, in particular by including patients with other chronic inflammatory diseases (e.g. patients with chronic rheumatoid arthritis).**

   We will add patients with other chronic inflammatory diseases such as rheumatoid arthritis to our future studies. Fortunately, there is a large rheumatoid arthritis research group at our institution, which allow access to these samples. We addressed this in the last paragraph of the discussion.

3. **To search for similar mutations in neurons and glia of the MS brain to establish a link to neurodegeneration in this disease.**

   Our ongoing studies will apply the techniques used in this manuscript in central nervous system tissues isolated from MS patients at autopsy. This is addressed in paragraph 6 of the discussion. As we complete these studies, as well as studies examining the role of SNVs on the immune response in MS patients, this will address Professor Lassmann's concern about these findings being relevant to the pathogenesis of MS.

4. **Professor Lassmann also suggested that oxidative stress might be a cause of SNVs in the PBMCs of MS patients.**

   We added this insight with references to the fourth paragraph of the discussion: "MS is also characterized by increased oxidative stress (in PBMC and brain), which can cause DNA damage and somatic mutations."

Competing Interests: No competing interests.