Analysis of Nuclear Export Sequence Regions of FUS-Related RNA-Binding Proteins in Essential Tremor

Oswaldo Lorenzo-Betancor¹, Kotaro Ogaki¹, Alexandra Soto-Ortolaza¹, Catherine Labbé¹, Carles Vilarino-Güell², Alex Rajput³, Ali H. Rajput³, Pau Pastor³,⁴,⁵, Sara Ortega⁴,⁵, Elena Lorenzo⁴,⁵, Audrey J. Strongosky⁷, Jay A. van Gerpen⁷, Ryan J. Uitti⁷, Zbigniew K. Wszolek⁷, Owen A. Ross¹,⁸

Introduction

Essential tremor (ET) is the most common movement disorder of the elderly and is characterized by a postural or motion tremor [1]. Recently, exome sequencing in a large pedigree with an autosomal dominant form of familial ET proposed a rare mutation in the nuclear export signal (NES) region of the FUS gene [1]. Other studies have shown that mutations in the FUS gene cause familial amyotrophic lateral sclerosis (ALS) [3–10]. Moreover, mutations in heterogeneous nuclear ribonucleoprotein A1 (hnRNP A1; OMIM*600124) and hnRNP A2/B1 (hnRNP A2/B1; OMIM*600124) have been described in families with multisystem proteinopathy and ALS [11]. TATA box-binding protein-associated factor 2N (TAF15; OMIM*601574) and Ewing sarcoma breakpoint region 1 (EWSR1; OMIM*137070) have been implicated in neurodegenerative diseases. For example, mutations in TAR DNA binding protein (TARDBP; OMIM*164017) and FUS cause familial amyotrophic lateral sclerosis (ALS) [3–10].

Abstract

Background and Objective: Genes encoding RNA-binding proteins, including FUS and TDP43, play a central role in different neurodegenerative diseases such as amyotrophic lateral sclerosis and frontotemporal lobar degeneration. Recently, a mutation located in the nuclear export signal (NES) of the FUS gene has been reported to cause an autosomal dominant form of familial Essential tremor.

Material and Methods: We sequenced the exons coding the NES domains of five RNA-binding proteins (TARDBP, hnRNP A2/B1, hnRNP A1, TAF15 and EWSR1) that have been previously implicated in neurodegeneration in a series of 257 essential tremor (ET) cases and 376 healthy controls. We genotyped 404 additional ET subjects and 510 healthy controls to assess the frequency of the EWSR1 p.R471C substitution.

Results: We identified a rare EWSR1 p.R471C substitution, which is highly conserved, in a single subject with familial ET. The pathogenicity of this substitution remains equivocal, as DNA samples from relatives were not available and the genotyping of 404 additional ET subjects did not reveal any further carriers. No other variants were observed with significant allele frequency differences compared to controls in the NES coding regions.

Conclusions: The present study demonstrates that the NES domains of RNA-binding proteins are highly conserved. The role of the EWSR1 p.R471C substitution needs to be further evaluated in future studies.

Citation: Lorenzo-Betancor O, Ogaki K, Soto-Ortolaza A, Labbé C, Vilarino-Güell C, et al. (2014) Analysis of Nuclear Export Sequence Regions of FUS-Related RNA-Binding Proteins in Essential Tremor. PLoS ONE 9(11): e111989. doi:10.1371/journal.pone.0111989

Supporting Information files.

Data Availability: The authors confirm that all data underlying the findings are fully available without restriction. All relevant data are within the paper and its Supporting Information files.

Funding: This work was supported in part by a Morris K. Udall Parkinson’s Disease Research Center of Excellence (NINDS P50 NS072187) and the Spanish Ministry of Economy and Competitivity grants SAF2006-10126 (2006-2009) and SAF2010-22329-C02-01 (2011-2013) to PP and by the UTE project FIMA to PP and SOC and by the Canada Research Chair program. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist. OAR is a PLOS ONE Editorial Board member. This does not alter the authors’ adherence to PLOS ONE Editorial policies and criteria.

* Email: ross.owen@mayo.edu
Figure 1. Nuclear export signal (NES) prediction of candidate proteins based on the NetNES 1.1 prediction tool [16]. NN = neural network algorithm; HMM = hidden Markov Model algorithm; NES score = combination of NN and HMM algorithms; QGSY-rich = glutamine, glycine, serine, tyrosine rich region; G-rich = glycine rich region; RRM = RNA recognition motif; RGG = Arg-Gly-Gly rich domain; Zn = zinc finger domain; The ? denotes that the NES predicted location does not surpass the NetNES established threshold. The thin black line denotes the prion-like domain location and the thick black line represents the highest score core region according to the Alberti algorithm [20].

doi:10.1371/journal.pone.0111989.g001
Table 1. Demographic data of Discovery and Replication Samples.

|                        | Discovery Sample | Replication Samples | Replication Sample 2 (Spain) | Whole Sample |                  |
|------------------------|-----------------|---------------------|-----------------------------|--------------|-----------------|
|                        | USA             | Replication Sample 1 (Canada) | Replication Sample 2 (Spain) |               | Whole Sample    |
| Fam ET                 | (n = 151)       | (n = 113)           | (n = 291)                   | (n = 328)    | (n = 39)       |
| Spo ET                 | (n = 106)       | (n = 178)           | (n = 39)                    | (n = 74)     | (n = 113)      |
| All ET                 | (n = 257)       | (n = 118)           | (n = 328)                   | (n = 401)    | (n = 182)      |
| Cont                   | (n = 376)       | (n = 291)           | (n = 39)                    | (n = 74)     | (n = 113)      |
| Age* (SD)              | 74.28 (11.66)   | 71.76 (15.08)       | 60.00 (15.10)               | 71.54 (14.20) |
|                        | 75.77 (11.76)   | 74.91 (12.93)       | 65.96 (14.55)               | 73.30 (13.56) |
|                        | 74.90 (11.83)   | 73.65 (13.92)       | 64.88 (15.01)               | 72.48 (13.89) |
|                        | 67.12 (12.26)   | 73.26 (12.37)       | 68.92 (10.43)               | 69.63 (12.25) |
| Age* range             | 35–96           | 23–97               | 19–88                       | 19–99        | 19–99          |
| AAO* (SD)              | 47.04 (19.91)   | 52.10 (19.06)       | 49.77 (18.83)               | 49.21 (19.61) |
|                        | 56.10 (18.37)   | 56.33 (16.39)       | 53.53 (20.87)               | 56.16 (18.12) |
|                        | 50.66 (19.45)   | 54.74 (17.56)       | 53.86 (20.43)               | 53.00 (19.12) |
| AAO* range             | 5–84            | 12–87               | 10–89                       | 5–87         | 6–89           |
| Female, %              | 54.31           | 53.31               | 53.35                       | 54.46        | 60.38          |

Fam ET = familial essential tremor; Spo ET = sporadic essential tremor; Cont = Healthy controls; y = years; AAO = age at onset; SD = standard deviation; NA = data not applicable.

*Age was not available for 57 subjects (6 familial, 17 sporadic cases and 34 controls) from Replication Sample 1 and for 11 subjects (1 familial, 3 sporadic cases and 7 controls) from Replication Sample 2.

AAO was not available for 22 subjects (10 familial and 12 sporadic cases) from the Discovery Sample, for 27 subjects (14 familial and 13 sporadic cases) from the Replication Sample 1 and for 6 subjects (4 familial and 2 sporadic cases) from Replication Sample 2.

doi:10.1371/journal.pone.0111989.t001
Figure 2. Conservation of EWSR1 p.R471 amino acid. Species alignment of EWSR1 p.R471 amino acid showing its highly preservation across evolution and the exact location of p.R471C substitution (rs138287627). Picture extracted from the UCSC Genome Browser (http://genome.ucsc.edu/cgi-bin/hgGateway).

doi:10.1371/journal.pone.0111989.g002

Our previous sequencing studies of the entire coding region of FUS gene in ET did not identify any additional pathogenic variants within the NES domain [3,4]. In the present study we have sequenced the predicted NES locations of these additional RNA-binding proteins in a cohort of ET subjects and controls, in order to identify novel mutations in those regions that may be responsible for disease.

Materials and Methods

All individuals gave their written informed consent and the study was approved by the Mayo Clinic Institutional Review Board, Jacksonville, Florida and the respective local Ethical Committees.

The Mayo Clinic ET series includes 257 patients, 151 individuals with familial ET and 106 sporadic ET subjects (Table 1). A diagnosis of ET was established according to the Consensus Statement of the Movement Disorder Society on Tremor [15] by an experienced neurologist specialized on movement disorders [JAvG, RJU and ZKW]. A series of 376 healthy subjects from Mayo Clinic, Jacksonville, was sequenced to establish the minor allele frequency (MAF) of identified mutations in a control population. All participants in the study are unrelated, non-Hispanic Caucasians recruited at Mayo Clinic, Jacksonville. In order to replicate the results of our study, we further genotyped two additional ET series and matched healthy controls to establish the frequency of the EWSR1 p.R471C substitution which is located in a highly conserved region of the protein (Figure 2). We genotyped 291 Canadian ET patients and 328 matched healthy subjects and 113 Spanish ET patients and 182 matched healthy subjects from Mayo Clinic, Jacksonville, was sequenced to establish the minor allele frequency (MAF) of identified mutations in a control population. All participants in the study are unrelated, non-Hispanic Caucasians recruited at Mayo Clinic, Jacksonville. In order to replicate the results of our study, we further genotyped two additional ET series and matched healthy controls to establish the frequency of the EWSR1 p.R471C substitution which is located in a highly conserved region of the protein (Figure 2). We genotyped 291 Canadian ET patients and 328 matched healthy subjects and 113 Spanish ET patients and 182 matched healthy subjects (Table 1).

The NetNES prediction server (http://www.cbs.dtu.dk/services/NetNES/) [16] was used to identify potential NES signals in the candidate genes. This online tool works with amino acid sequences and combines both neural networks (NN) and hidden Markov models (hMM) in its prediction algorithm. The integration of both models allows us to combine the superior observed specificity of the hMM with the observed superior sensitivity of the NN [16]. Once the NES amino acid signals were identified (Figure 1), we performed bidirectional sequencing of the exons of each gene coding for these specific regions (see Table S1 for specific primers).

Variants were numbered according to standard nomenclature based on RefSeq mRNA and peptide accession numbers for each gene (TARDBP: NM_007375.3, NP_031401.1; hnRNPA1: NM_002136.2, NP_002127.1; hnRNPA2B1: NM_031243.2, NP_112533.1; TAF15: NM_139215.2, NP_631961.1; EWSR1: NM_005243.3, NP_005234.1). PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/index.shtml) and SIFT (http://sift.bii.a-star.edu.sg/) algorithms were used to assess the impact of the amino acid substitutions on the protein structure. The virtual effect of intronic variants on splicing was assessed using the Human Splicing Finder (HSF) algorithm (http://www.umd.be/SSF/) [17]. Allelic association analysis and Bonferroni correction for multiple testing were performed with PLINK v.1.07 software (http://pngu.mgh.harvard.edu/purcell/plink/) [18].

Results

Our sequencing analysis of 257 patients with ET did not identify any novel variants in the predicted NES coding regions of the candidate genes. We identified two previously described missense variants in the EWSR1 gene (rs41311143, p.G465S and rs138287627, p.R471C; Table 2), which are predicted to be probably damaging and benign by the Polyphen-2 algorithm, respectively. The minor allele frequency (MAF) of the EWSR1 p.G465S variant was the same in ET cases and controls (MAF 1.5%) whereas the p.R471C substitution was observed in only one patient with autosomal dominant familial ET. Unfortunately, DNA from the rest of the family members was not available to asses for co-segregation of this variant with disease. The genotyping of 404 additional ET patients and 510 healthy controls did not reveal any additional EWSR1 p.R471C carriers.

Across the candidate genes we observed a known synonymous variant in the TARDBP gene (rs147795017, p.Y374Y; Table 2) and eight intronic variants, two of which were novel (hnRNPA2B1 c.42+17T>C and EWSR1 c.1417+68_1417+71delGATT; Table 2). The hnRNPA2B1 c.42+17T>C mutation was present in a single sporadic case and was absent in a series of 376 healthy controls. The HSF algorithm predicts this mutation to change the exon 2 splicing site including 15 intronic nucleotides between exons two and three, which would cause an in-frame insertion of five amino acids (VLQQQ), but RNA from the mutation carrier was not available for examination. The EWSR1 c.1417+68_1417+71delGATT variant was present in three ET
Table 2. Genotype counts of variants identified in predicted NES regions of candidate genes from the Discovery Sample.

| Gene | Location | rs number | Mutation | AA change | Major Het Minor MAF | Major Het Minor MAF | EVS MAF | 1 kG MAF | P value * | PolyPhen-2 | SIFT score |
|------|----------|-----------|----------|-----------|---------------------|---------------------|---------|---------|-----------|-------------|------------|
| EWSR1 | Int 10 | rs41309649 | c.1046–17C>G | Intronic | 250 7 0 | 0.014 | 366 8 0 | 0.010 | 0.01 | 0.63 | NA | NA |
| EWSR1 | Int 11 | rs3761426 | c.1164+37T>G | Intronic | 186 63 8 | 0.154 | 263 90 21 | 0.120 | 0.12 | 0.29 | NA | NA |
| EWSR1 | Ex 13 | rs41311143 | c.1393G>A | p.G465S | 250 7 0 | 0.014 | 358 11 0 | 0.015 | 0.01 | 0.85 | Tolerated (0.96) |
| EWSR1 | Ex 13 | rs138287627 | c.1411C>T | p.R471C | 256 1 0 | 0.002 | 369 0 0 | 0.000 | 7.68 x 10^-5 | NA | 0.23 | Benign (0.013) |
| EWSR1 | Int 13 | rs3747142 | c.1417+51A>G | Intronic | 186 68 5 | 0.152 | 258 103 8 | 0.137 | 0.12 | 0.65 | NA | NA |
| EWSR1 | Int 13 | NA | c.1417+68_1417+71delGATT | Intronic | 252 3 0 | 0.006 | 361 8 0 | 0.011 | NA | NA | 0.36 | NA | NA |
| hnRNPA1 | Int 1 | rs2071391 | c.16-57G>A | Intronic | 186 59 7 | 0.145 | 265 103 8 | 0.137 | NA | 0.24 | 0.51 | NA | NA |
| hnRNPA2B1 | Int 2 | NA | c.42+17T>C | Intronic | 256 1 0 | 0.002 | 375 0 0 | 0.000 | NA | NA | 0.23 | NA | NA |
| hnRNPA2B1 | Int 3 | rs41275982 | c.153+4T>C | Intronic | 242 15 0 | 0.029 | 355 31 1 | 0.025 | 0.03 | 0.03 | 0.90 | NA | NA |
| TAF15 | Int 12 | rs4251774 | c.1006+34A>G | Intronic | 247 10 0 | 0.019 | 373 3 0 | 0.004 | 0.01 | 0.01 | 0.01 | NA | NA |
| TARDBP | Ex 6 | rs147795017 | c.1122T>C | p.Y374Y | 255 1 0 | 0.002 | 365 0 0 | 0.000 | 7.7 x 10^-3 | NA | 0.23 | Tolerated (1) |

Genes are sorted in alphabetical order. ET = essential tremor; AA = Amino acid; EVS = Exome Variant Server; 1 kG = 1000 Genomes; MAF = minor allele frequency; Ex = exon; Int = intron; Het = count of heterozygous carriers; NA = not applicable; SIFT = Sorting Tolerant From Intolerant algorithm.

*Uncorrected p-value calculated with PLINK case-control association analysis.

doi:10.1371/journal.pone.0111989.t002
Discussion

The involvement of mutated RNA-binding proteins in several neurodegenerative disorders suggests that this family of proteins may be relevant across heterogeneous disease phenotypes. The identification of a nonsense mutation in the NES domain of the FUS protein (p.Q290X) in a large kindred with autosomal dominant ET has raised interest in the role of these genes in this common movement disorder. In the present study we screened the predicted NES regions of other RNA-binding proteins that have been associated with neurodegeneration but did not identify any novel variants related to the ET phenotype.

FUS mutations have been proposed to be involved both in ALS [5,7] and in ET [2]. However, the described mutations in both diseases are located in different domains of the protein. While the ALS mutations affect the RGG domain [7], the mutation causing ET results in a premature stop codon located in the NES region of the protein [2]. Additionally, functional analyses have shown that the pathogenic effect of the ET-specific FUS mutation, whose mRNA is degraded by the nonsense-mediated decay (NMD) pathway, differ from those of the ALS mutations, whose mRNAs do not undergo this kind of degradation [2]. This fact suggests that the affected domain of the protein and type of mutation plays a critical role in determining the disease phenotype developed by the affected domain of the protein and type of mutation plays a critical role in determining the disease phenotype.

A recent study has shown that ~1% of human protein-coding genes contain a potential prion-like domain and of this 1%, there is a 12-fold enrichment from proteins containing also a canonical

References

1. Puschmann A, Wzorek ZK (2011) Diagnosis and treatment of common forms of tremor. Semin Neurol 31: 63–77.
2. Merker ND, Girard SL, Casoare B, Bourassa CV, Belati VV, et al. (2012) Exome sequencing identifies FUS mutations as a cause of essential tremor. Am J Hum Genet 91: 313–319.
3. Labbe C, Soto-Oroñez AI, Rayaprolu S, Harriott AM, Strongosky AJ, et al. (2013) Investigating the role of FUS exon variants in essential tremor. Parkinsonism Relat Disord 19: 755–757.
4. Ortega-Cubero S, Lorenzo-Betancor O, Lorenzo E, Alonso E, Coria F, et al. (2013) Fused in Sarcoma (FUS) gene mutations are not a frequent cause of essential tremor in Europeans. Neurobiol Aging 34: 2441 e2449–2441 e2411.
5. Vance C, Rogelj B, Hortschagbi T, De Vos KJ, Nishamura AL, et al. (2009) Mutations in FUS, an RNA processing protein, cause familial amyotrophic lateral sclerosis type 6. Science 323: 1208–1211.
6. Neumann M, Sampathu DM, Kwong LK, Truax AC, Micsenyi MC, et al. (2006) Ubiquitinated TDP-43 in frontotemporal lobar degeneration and amyotrophic lateral sclerosis. Science 314: 130–133.
7. Kwiatkowski TJ Jr, Bosco DA, Leclerc AI, Tamrazian E, Vanderburg CR, et al. (2009) Mutations in the FUS/TLS gene on chromosome 16 cause familial amyotrophic lateral sclerosis. Science 323: 1205–1208.
8. Da Cruz S, Cleveland DW (2011) Understanding the role of TDP-43 and FUS/TLS in ALS and beyond. Curr Opin Neurobiol 21: 904–919.
9. Chea-Plotkin AS, Lee VM, Trojanowski JQ (2010) TAR DNA-binding protein 43 in neurodegenerative disease. Nat Rev Neurol 6: 211–220.
10. Arai T, Hasegawa M, Akiyama H, Ikeda K, Nonaka T, et al. (2006) TDP-43 is a component of ubiquitin-positive tau-negative inclusions in frontotemporal lobar degeneration and amyotrophic lateral sclerosis. Biochem Biophys Res Commun 351: 602–611.
11. Kim HJ, Kim NC, Wang YD, Scarborough EA, Moore J, et al. (2013) Mutations in prion-like domains in hnRNPA2B1 and hnRNPA1 cause multisystem proteinopathy and ALS. Nature 495: 467–473.
12. Coutinho J, Hart MP, Shorter J, DeJesus-Hernandez M, Erion R, et al. (2011) A yeast functional screen predicts new candidate ALS disease genes. Proc Natl Acad Sci U S A 108: 20891–20896.
13. Neumann M, Bentmann E, Dormann D, Javitt D, DeJesus-Hernandez M, et al. (2011) FUS proteins TAF15 and EWS are selective markers that distinguish FTLD with FUS pathology from amyotrophic lateral sclerosis with FUS mutations. Brain 134: 2593–2609.
14. Tisci N, Vauze G, Leclerc AI, Keagle P, Glass JD, et al. (2011) Mutational analysis reveals the FUS homolog TAF15 as a candidate gene for familial amyotrophic lateral sclerosis. Am J Med Genet B Neuropsychiatr Genet 156B: 285–290.
15. Druschl G, Bain P, Brin M (1998) Consensus statement of the Movement Disorder Society on Tremor. Adv Hoc Scientific Committee. Mov Disord 13 Suppl 3: 2–23.
16. la Cour T, Kiemer L, Molgaard A, Gupta R, Skriver K, et al. (2004) Analysis of DNA samples from affected relatives, nor its presence in other ET populations, the EWSR1 p.R471C substitution is a candidate variant that needs to be further screened in future ET studies.

Supporting Information

Table S1 Sequencing primers. (DOCX)

Acknowledgments

We wish to thank the patients and families who participated in the study.

Author Contributions

Conceived and designed the experiments: OLB KO ASO CL AR. Performed the experiments: OLB KO ASO CL CVG SO EL AJS JVG RU ZKW OAR. Analyzed the data: OLB KO CVG PP OAR. Contributed reagents/materials/analysis tools: OLB KO ASO CL AR CVG AHR AJS PP JVG RU ZKW OAR. Contributed to the writing of the manuscript: OLB OAR. Critical Revision of manuscript: KO ASO CL CVG PP AJS JVG RU ZKW.

Disclosure

This research was supported by the following grants: National Institute of Health/National Institute of Neurological Disorders and Stroke (NINDS) 1R01NS079001-01, 5R01NS060412-04, 1R01NS081834-01, and 5R01NS087103-04. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institute of Health/National Institute of Neurological Disorders and Stroke (NINDS).